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14. ABSTRACT
This proposal addresses the intent of the NIH CounterACT Program to reduce mortality and morbidity resulting from the release of chemical threats such as CN. Since the onset of CN toxicity is rapid, the prognosis of a victim depends on the immediate and aggressive application of a safe, efficacious, rapidly acting, and easily administered antidote. The major purpose of this project is to develop an efficient, easy to administer (i.e., intramuscular; IM) sulfur-donor-based CN countermeasure, which may also be used in combination with other countermeasures (e.g., cobinamide and sulfanegen, which are currently under development). Preliminary in vivo efficacy studies from our laboratory indicate that dimethyl trisulfide (DMTS), a naturally occurring component of garlic and generally recognized as safe as a flavor enhancer by the FDA, is a promising SD effective in countering CN toxicity.

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ABBREVIATIONS

DMTS= Dimethyl trisulfide; mDMTS = micellar DMTS; Poly80 = Polysorbate 80; SPME= Solid phase micro extraction; Cbi= Cobinamide;

Section I. Introduction/Rationale

This proposal addresses the intent of the NIH CounterACT Program to reduce mortality and morbidity resulting from the release of chemical threats such as CN. Since the onset of CN toxicity is rapid, the prognosis of a victim depends on the immediate and aggressive application of a safe, efficacious, rapidly acting, and easily administered antidote. The major purpose of this project is to develop an efficient, easy to administer (i.e., intramuscular; IM) sulfur-donor-based CN countermeasure, which may also be used in combination with other countermeasures (e.g., cobinamide and sulfanegen, which are currently under development). Preliminary *in vivo* efficacy studies from our laboratory indicate that dimethyl trisulfide (DMTS), a naturally occurring component of garlic and generally recognized as safe as a flavor enhancer by the FDA, is a promising SD effective in countering CN toxicity.

Specific Aims

1. Development of an optimized IM SD formulation. This specific aim addresses the formulation development for DMTS, or in consultation with NIH, other molecules of interest, alone and in combination with other countermeasures. Specific issues for the IM dosage form (e.g. biocompatibility, viscosity, toxicity) will be addressed during the formulation development.

2. Determining *in vivo* efficacy of DMTS for each selected formulation, using a mouse model. This portion of the project will include the determination of antidotal potency ratios (APR) as a ratio of LD_{50} of CN (with antidotes)/LD_{50} of CN (without

antidotes) for the DMTS formulations, applied alone or/and in combination with other countermeasures (e.g. cobinamide, or sulfanegen) using IM administration.

Section II. Research Report

II.1. Year 01 Milestone #1: Demonstrate appropriate formulation efficacy and stability *in vitro* and *in vivo,* achieving efficient absorption kinetics and bioavailibity with the developed nano-disperse formulations of the potential therapeutical agents: DMTS alone, and in combination with other CN antidotes. Demonstrate initial *in vivo* safety. *Success:* Degradation within allowed limits or no degradation during storage at room temperature (weeks), or in refrigerator (months). Formulated drugs can successfully cross cell membranes, exhibit fast absorption (in minutes), and 50% persistence in the circulation for at least half an hour, achieve bioavailability of 65% or higher (IM administration). Acceptable or no detectable adverse effects alone and in combination with cobinamide at the doses applied. *Rationale:* By developing a more stable, IM administered CN antidote, with a shelf-life equal to or greater than currently available CN antidotes, our preparedness for treating victims in a mass casualty situation will be enhanced. (Specific Aim 1)

☐ **Met** ☐ **Not Met** ☒ **In Progress**

II.1.1. Formulation Development for DMTS alone / Stability Studies

II.1.1.1. Micellar formulation with DMTS (mDMTS) and Stability / Efficacy Studies with mDMTS

Background:

Micelles represent and offer an attractive avenue to developing a carrier system for the lipophilic DMTS molecule. Micelles are spherical structures composed of a hydrophobic core and a hydrophilic corona with sizes ranging from 5 - 50 nm. They are produced by hydrating films of block co-polymers like PEG-PE. Instead of forming bilayers and subsequent liposomes, the unique structure of block co-polymers allow them to partition into a hydrophobic phase consisting of the fatty acid tails of the phospholipids surrounded by the hydrophilic groups consisting of the PEG and phosphate groups. Pegylated micelles have been proposed and used as carriers of hydrophobic anticancer drugs like paclitaxel. Therefore, the aim of the present study was to prepare and characterize micelles encapsulating DMTS (mDMTS) which would simultaneously ensure high concentrations of the sulfur donor and avoid tissue damage after parenteral intramuscular administration. It was also our purpose to determine the solubility of DMTS in the prepared carriers, examine its stability, and optimize the micelle preparation and composition so that it can serve as an appropriate DMTS vehicle for the *in vivo* antidotal efficacy studies. The study also aimed to determine both the *in vitro* and *in vivo* efficacy of DMTS, as a new, potential therapeutic agent to combat cyanide intoxication. Thus, it was our aim to determine the CN to SCN conversion by the antidote candidate over a wide range of concentrations and establish its therapeutic efficacy.

Optimization of micelle preparation technology

DMTS exhibits very poor water solubility thus an appropriate vehicle, namely a micelle composition had to be developed for the *in vivo* studies. Prior to solubilizing DMTS in the micelles, its preparation technology was optimized dividing the process into 5 steps. An important finding of the optimization studies was that that DMTS should not be added to the stock solution containing PEG_{2000}-DSPE in ethanol but should be added to the micelles following hydration. This technological step is crucial because as DMTS assays following the micelle preparation showed only a very low concentration of antidote was present in the micelle solution when DMTS was added to the initial solution. This phenomenon can be linked the enhanced evaporation/degradation of DMTS during the film formation step of the preparation. A second, equally important discovery was made during the optimization, namely that sonication at 50°C for 20 minutes also contributes to loss of DMTS; therefore, this manufacturing step should not be applied. Based on these findings an optimized technology is presented for the manufacture of micelles loaded with a liquid drug subject to evaporation/degradation on heating.

DMTS loaded micelle preparation, and CN conversion by mDMTS

Applying the optimized technology PEG_{2000}-DSPE and mixed micelles comprising PEG_{2000}-DSPE/TPGS (molar ratio 1:1) were prepared and maximum DMTS solubility was determined in all the samples (Figure 1). It was revealed that 1) as the concentration of the micelles increased so did the concentration of solubilized DMTS, 2) PEG_{2000}-DSPE exhibited a superior solubility enhancing effect compared to the mixed

micelles at all examined concentrations. Highest solubility was seen at 26.73 mM PEG_{2000}-DSPE concentration where a maximum DMTS solubility of 2.5 mg/ml was reached. Although further solubility enhancement was expected at higher micelle forming agent concentration, due to the high cost of these excipients this would not be advantageous.

Investigating mDMTS by SPME-GC-MS

During manufacturing processes it was noted that DMTS might volatilize. It was considered that alongside the beneficial solubility enhancing effect of the micelles they might also decrease the rate of volatilization. The micelles were efficient in stabilizing the volatile DMTS (boiling point - 58ºC) and proved to hinder the volatilization of DMTS better than plain DMTS in alcohol when incubated at 37ºC. The amount of DMTS in the head space remains constant for almost two hours in the case of mDMTS whereas with DMTS in alcohol it rapidly declines and after eight hours has declined to about 60% of the original levels. Further test have to be performed to determine the long term stability of the preparation.

Histopathology of mouse tissue after intramuscular injection of mDMTS

The three DMTS treatments were similar across dosages and were similar across time points. Time points demonstrated an initial change (4 h) of muscle swelling, degeneration, and fragmentation accompanied by very mild edema and fibrin and neutrophil infiltration. By 8 h macrophages were observed as part of the inflammatory cell infiltrate. By 12 and 24 hours the edema and inflammation had increased to some degree and there was evidence of satellite cell hyperplasia at the periphery of some degenerating fibers (initial attempts at regeneration). The untreated (negative) control

had no significant lesions within the muscle. The treated (positive) control of 10 mM phosphate buffer had acute degenerative and inflammatory lesions similar to those observed with each treatment at the 8- hour time point. All changes were interpreted to be most likely due to trauma/pressure associated with an intramuscular injection, and not toxic effects of the material injected.

Conclusion of mDMTS Formulation

To overcome the deficiency of the presently available CN antidotes of Nithiodote and Cyanokit, (intravenous administration, methemoglobinemia by sodium nitrite, poor sulfur donor efficiency and poor cell penetration capability and high Rh dependency of thiosulfate), a series of organo-sulfur molecules have been tested as sulfur donors. Some of them proved to be superior to the present therapy of thiosulfate (more efficient sulfur donor reactivity and higher lipohilicity), and the choice was given to DMTS for further investigations. DMTS proved to be a significantly more efficient sulfur donor than the present therapy of sodium thiosulfate, and it reacts efficiently with CN even without Rh, therefore it seems to be an appropriate candidate for developing an intramuscular injection kit, usable for a mass casualty scenario. Furthermore, DMTS is a known, naturally occurring molecule: it is present in garlic, and is used in food industry as flavor enhancer, therefore it seems to be a safe candidate molecule.

These studies are the first to prove that DMTS is efficacious after intramuscular administration, underlining the findings reported with MPTS, namely that adsorption from the muscle is rapid enough to counteract CN intoxication (Kovacs et al., 2013). This is an important finding because it shows that a future antidote kit could be formulated as intramuscular product. This would have numerous advantages, such as

self-administration, easier handling and distribution in a mass casualty scenario over the currently approved kits which can only be administered intravenously. The use of micelles, proposed in this paper does not fully solve the solubility issues of DMTS, but is a valuable initial step in reaching an adequate formulation. The advantages of mDMTS vs. un-encapsulated DMTS are: 1) elimination of muscle necrosis, 2) the rate of evaporation within mDMTS is suppressed, that can provide a level of stability for the formulation. Two types of micelles (PEG_{2000}-DSPE and PEG_{2000}-DSPE/TPGS) were prepared and tested for their ability to encapsulate DMTS. The method of micelle preparation for the liquid drug, DMTS was optimized and it was demonstrated that the PEG_{2000}-DSPE preparation can dissolve up to 2.5 mg/ml of the antidote candidate. However, keeping it in consideration that the injection volume has to be kept minimized, with this mDMTS a maximum dose of 12.5 mg/kg DMTS can be applied. However, even this low dose of DMTS showed a remarkable *in vivo* therapeutic efficacy of 2 X LD_{50} protection in a mice model. When DMTS load of higher than 2.5 mg/ml was applied to the micelle forming excipients, a mixture of micelles and emulsions were seen, that gave higher *in vivo* efficacy, but due to the lack of the physical stability of the composition and standardized process parameters the formulation could not be further tested (Petrikovics unpublished data).

Summarizing the work, it can be concluded that the *in vitro* and *in vivo* findings proved the efficacy of DMTS in combating CN intoxication and the presented work gives valuable insight to micelle preparation and sets the bases for a future formulation of DMTS. However, for applying standardized higher DMTS dose, further development of advanced formulations is necessary.

II.1.1.2. Formulations based on Cyclodextrines, Co-Solvents and Surfactants:

First attempt focused on a micellar encapsulation of DMTS. For intramuscular administration, the injection volumes need to be minimized. With the micellar encapsulation, the maximum solubility of 2 mg/ml was achieved, that made a limitation of the applied dose of DMTS as maximum of 12.5 mg/kg. However, this low dose of DMTS was proved to protect 2 X LD50 against CN therapeutically. It was expected, that higher DMTS dose would provide higher therapeutic antidotal protection. Therefore further investigation efforts were focused on developing formulations for DMTS, which can be applied in higher does.

Present study describes the solubility studies with various cyclodextrins, surfactants, co-solvents and their combinations and the *in vivo* efficacy of the antidote tested with the developed formulation.

Solubility of DMTS in cyclodextrins

The solubility of DMTS in the examined, FDA approved cyclodextrin solutions is shown in Figure 2. The solubility of a drug versus cyclodextrin concentration graph is indicative of the solubilizing effect of the utilized cyclodextrin and can be classified into two major types: A and B. (Brewster and Loftsson, 2007; Del Valle, 2004). All the tested cyclodextrins exhibit A_L type graphs meaning that the solubility of DMTS increased linearly with the linear increase of cyclodextrin concentration. Both the solubility values and the graphs are indicative of the fact that the formed complexes are more soluble than the uncomplexed antidote candidate. The highest DMTS concentration, namely 11.69 mg/ml was seen with 0.12M SBE-β-CD yielding a 90 fold increase in solubility

compared to the molecule's water solubility. Conclusion: although the DMTS formulation with cyclodextrin-derivatives increased the solubility of DMTS substantially it did not provide high enough concentration to IM injection.

Solubility of DMTS in co-solvents

The effect of co-solvents on the solubility of DMTS was examined using ethanol, polyethylene glycols PEG 200 and PEG 300, propylene glycol at concentrations of 10%, 25%, 50%, 75% and 90% (Figure 3). It has been previously reported that the solubility enhancing effect of co-solvents follow a log-linear type graph (Yalkowsky at al., 1972, Yalkowsky et al., 1976), which pattern was also seen on the solubility graph of DMTS (figure insert). The concentration of the SD at 90%, 75% and 50% ethanol are 200.5 ± 31 mg/mL, 60.5 ± 0.4 mg/mL and 10.9 ± 1.5 mg/mL respectively. These values represent a 1538, a 461 and an 84 fold increase in the solubility of the molecule compared to its water solubility (0.13 mg/mL). The second and third most effective solubilizers PEG200 and PEG 300 possess similar solubilizing capacities both dissolving ≈40 mg/mL and ≈7.5 mg/mL DMTS at 90% and 75% respectively, increasing the SD's solubility by 307 and 58 fold. Although the solubility of DMTS was also increased at lower concentrations, the dissolved amount is not relevant in aspect of the studies that followed. Based on the solubility results in co-solvent/water systems co-solvent/co-solvent/water systems were prepared. It was seen that the 10% systems did not increase the solubility of DMTS by a considerable amount thus in the further studies this concentration of excipients was no longer tested. The 90% systems were no longer tested due to the expected toxicity associated with their high concentration. The

solubility of the antidote candidate in co-solvent/co-solvent/water mixtures is presented in Figure 4.

Combination studies performed with ethanol and various co-solvents show that the solubility of DMTS in these systems does not exceed the solubility in ethanol, but the combination of the excipients in one solvent system might still be advantageous because the concentration of ethanol can be effectively decreased while still maintaining solubility values close to the ones measured in ethanol.

Based on these results it became obvious that using a solvent system comprising solely of co-solvents for the *in vivo* studies was not possible because of the high excipient concentrations needed to solubilize the molecule. This would probably lead to tissue damage during administration therefore, other solubilizers were also tested. Further studies were necessary to optimize the DMTS formulation usable for IM injection.

Solubility of DMTS in surfactants

The effect of surfactants on the solubility of DMTS was examined using Cremophor EL, Cremophor RH40, polysorbate 80, sodium cholate, sodium deoxycholate and Cremophor EL:Cremophor RH40 (ratio=1:1), Cremophor EL:polysorbate 80 (ratio=1:1) and Cremophor RH40:polysorbate 80 (ratio=1:1) at 1%, 5%, 10%, 15% and 20% (Figure 5). These concentrations are all above the critical micellar concentration of the excipients thus solubilization is achieved by the formed micelles (cmc values: Cremophor EL = 0.002%, Cremophor RH40 = 0.039%, polysorbate 80 = 0.016%, sodium cholate = 0.388-0.603%, sodium deoxycholate = 0.083-0.249%) (Coello et al., 1996; McBain, 1913; Rowe et al., 2009). The solubility enhancing effect of the

surfactants increased as the concentration of the excipients increased. Of the tested surfactants polysorbate 80 was the most effective, increasing the solubility of DMTS to 95.6 ± 0.7 mg/mL at 20% (a 735 fold increase compared to water solubility). As in the case of co-solvents all other excipients increased the solubility of the drug, but contrarily to co-solvents the difference of solubilizing power between the most and the second most effective surfactant is not as marked. Similarly to the case encountered with co-solvents combination of surfactants did not result in solubility values exceeding that of the most effective non-combined surfactant, namely polysorbate 80. However, as with co-solvents the combined surfactants showed substantial solubilizing power and might be useful in decreasing the concentration of one specific surfactant in the solvent system. Comparing the solubilizing effect of surfactants to that of co-solvents and cyclodextrins it can be concluded that former are more effective at dissolving DMTS.

***Summary of Conclusion about Formulations with Cyclodextrines, Surfactants, Co-Solvents and the Combination of Both*:** Present study addressed the development of an intramuscular dosage form and the determination of the therapeutic antidotal ratio of a highly lipophilic, potent sulfur donor compound that could possibly be used as an antidote against CN poisoning. Previous studies showed that the detoxification of CN through conversion to less toxic thiocyanate is enhanced in the presence of an appropriate sulfur donor and rhodanese, an enzyme catalyzing the sulfur atom transfer. It was also previously shown that in the case of certain types of sulfur donors, the presence or absence of rhodanese does not play a role in the conversion rate. One such molecule is DMTS. This oily substance exhibits very poor water solubility thus

formulation studies applying 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt) (PEG2000-DSPE) and PEG2000-DSPE/TPGS with a molar ratio of 1:1 as micelle forming excipients were undertaken. The solubility of the antidote was increased to a maximum of 2.5 mg/mL in the presence of 26.73mM PEG2000-DSPE and *in vivo* animal studies were conducted upto doses of 25 mg/mL. It was proven that DMTS is a potent antidote in mice model, but due to the low final concentration of the composition one of the drawbacks of the compositions is the high injection volume. Another issue arising from the relatively low DMTS concentration in the micelles was that it was not possible to test the antidotal potency of the drug at a higher dose than 25 mg/kg (Kovacs et al., 2013). Due to the limitations of the micelle formulation a new composition was developed. Aqueous solutions of cyclodextrins, cosolvents and surfactant were tested at different concentrations and their ability to increase the solubility of DMTS was evaluated. It was concluded that out of the three types of excipients tested surfactants, polysorbate 80 increased the solubility of the SD by the largest rate. A very substantial, 735 fold solubility increase compared to water solubility was seen when using 20% polysorbate 80 as the solubilizer. Results of formulation studies offer an attractive composition for animal studies which could also be used as the base for a human intramuscular liquid preparation. For further studies, the 15% polysorbate 80 was chosen and employed for DMTS formulation (**15% Poly80-DMTS**), however, later we also explored the **(20% Poly80-DMTS)** formulation composition for stability and *in vivo* efficacy studies.

Conclusion of DMTS–Poly80 formulation: Generally it is important to well homogenize and equilibrate the Poly80 with water! This is a delicate colloid system, if it is not well

homogenized, later, after DMTS addition, precipitation can occur. *Reminder:* The double sealing storage method prevents from the oxidation, or other DMTS content decreases; while the Poly80 –water system preparation determines the precipitation formation!

II.1.2. *In vitro* Stability Studies with DMTS alone (Poly80-DMTS)

Based on the previous solubility studies with various FDA approved co-solvents/surfactants and their combinations, Poly80 was chosen as the optimal solvent system to dissolve the highly lipophilic DMTS. As Figure 6. shows, the higher Poly80 concentration solvent systems can dissolve higher amounts of DMTS. The higher than 20% Poly80 concentrations for *in vivo* studies are not recommended in order to reduce the amount of excipients injected into the body.

Stability studies with 15% and 20% Poly80 - DMTS (50 mg/ml)

With this formulation the injection volumes can be optimized (minimized) and the muscle necrosis at the injection site is minimized. The stability studies with 15%Poly80-DMTS have been performed at 3 temperatures (+4 ^{0}C, +20 ^{0}C, and +40^{0}C, at pH of 2,4,7,9, and 11 using diluted HCl and NaOH, and the stability studies with (20% Poly80-DMTS) as functions of temperature, time and type of storage container. Samples were taken at various time intervals (indicated on the figures); (Fig 7a-e). Samples prepared at pH=7, stored in refrigerator (+4 ^{0}C) showed optimal stability (98%) up to 31 days. It was also proven, that the double- sealed storage method helped to prevent DMTS from evaporation (Picture1). There was no oxidation product/metabolite detected in the samples analyzed by GC-MS and HPLC over one month. The calibration curve of the

present GC-MS method showed good correlation with that of the present HPLC method, therefore we relied on both data equally.

The stability of the two Poly80 formulations for DMTS was measured and compared to the original DMTS content at +4 ^{0}C in double sealed crimped container when each container was opened only once for the measurements (Picture1).

As Figure 8 shows, there were no significant differences between the two Poly80 solvent systems. The advantage of the Poly80 (20%) could be the following: It can dissolve more DMTS (Figure 6), that can allow achieving lower injection volume. However, to dissolve more DMTS in Poly80 requires longer and stronger vortexing to be sure to dissolve the DMTS, and the high excipients concentration is not favorable for *in vivo* administration.

Conclusion of the stability studies with formulated DMTS alone:

Present studies focused on the characterization of the storage stability of the Poly80-formated DMTS. Previous studies addressed the development of an intramuscular dosage form and the determination of the therapeutic antidotal ratio of the highly lipophilic, potent sulfur donor compound that could possibly be used as an antidote against CN poisoning. It was also shown that the detoxification of CN through the conversion to the less toxic thiocyanate is enhanced by Rh. However, certain types of sulfur donors, can efficiently convert CN to SCN without Rh. One such molecule is DMTS. This oily substance exhibits very poor water solubility, thus formulation studies were needed to enhance the solubility and make it ready to be injected intramuscularly. The first attempts were focused on applying 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt)

(PEG2000-DSPE) and PEG2000-DSPE/TPGS with a molar ratio of 1:1 as micelle forming excipients and the solubility of the antidote was increased to a maximum of 2.5 mg/mL. In the presence of 26.73mM PEG2000-DSPE, with this micellar formulation, a maximum dose of 25 mg/kg DMTS was achieved. Due to the limitations of the micelle formulation and the need for a higher DMTS dose application, new compositions were developed by applying various co-solvents, surfactants and their combinations. It was concluded that out of the three types of excipients tested the surfactant polysorbate 80 increased the solubility of the SD by the largest rate. Present studies compared the *in vitro* storage stability of the two Poly80-formulated DMTS systems (15% Poly80-DMTS (50mg/ml) and 20% Poly80-DMTS (50 mg/ml)). When the stability was measured at three temperatures (+4 ^{0}C, +20 ^{0}C, and +40 ^{0}C) it was confirmed that the loss of DMTS in the sampling was the result of evaporation effects rather than a chemical degradation. It was also found that light had no effect on the DMTS content. Based on the intense studies with Poly80-DMTS, we recommend to keep the DMTS samples in hermetically closed containers, stored in refrigerator, with the pH kept around neutral. (Petrikovics, unpublished data). Exploring the application of antioxidants, such as Na_2HSO_3, Na_2SO_3, thio-glyocolic acid, thio-lactic acid, thio-carbamid, cysteine, ascorbic acid are also recommended.

These stability and *in vivo* antidotal efficacy data confirm that DMTS is a good candidate for treating CN intoxication, and the Poly80 formulation provides an optimal solvent system to dissolve the lipophilic DMTS in the required concentration with minimized injection volume, applicable for intramuscular administration from a possible injector kit designed for a mass scenario.

II.1.3. Pharmacokinetics studies with DMTS alone (Poly80-DMTS)

II.1.3.1. Analytical Method development for DMTS determination in Blood

We report here the development of a preliminary sample preparation method that enables DMTS concentrations in blood to be determined using high performance liquid chromatography coupled with ultraviolet absorption spectroscopy (HPLC-UV). Samples were prepared from blood that was drawn from rats and spiked with DMTS. Once prepared, the DMTS content of samples was analyzed using HPLC-UV. Chromatogram peak areas for DMTS in each spiked standard were measured and used to prepare calibration curves. Calibration curves show a linear response for DMTS concentrations between 0.01 and 0.3mg/mL.

Chromatograms: When the formulated DMTS samples made with 15% w/w Polysorbate 80 (DMTS-Poly80) were injected to the HPLC column, a DMTS peak with a retention time of 9.5 minutes were detected. A representative chromatogram (cyclohexanone extract of a 0.75 mg/mL DMTS-Poly80 solution) is shown in Figure 9.

Calibration curve for DMTS in blood

The calibration curve for DMTS in blood is shown in Figure 10. The curve shows high degree of linearity. The standard deviation of the standard signals from the calibration line is found to be 2330. Based on this the limit of detection for DMTS via this method is estimated to be 0.018 mg/mL, and the limit of quantitation for DMTS is estimated to be 0.042 mg/mL. Thus this calibration curve provides a mechanism for determining DMTS in the range between 0.04 to 0.30 mg/mL. By taking the ratio of the area predicted by the calibration curve for 0.5 mg/mL DMTS in ethanolic solution, and the experimentally

obtained peak areas the cyclohexanone extraction method was found to yield a 2.3 % recovery of DMTS spiked into rat blood. This calibration curve for DMTS in rat blood shows a negative offset from the origin. We hypothesize that both the low recovery and the nonzero intercept are indicators of analyte losses in the cyclohexanone extraction based sample preparation method. DMTS is a volatile molecule, and also a hydrophobic molecule. Losses at low concentrations may arise because of evaporation or because of partitioning to container surfaces. During early stage of the method development, we were interested in plasma portion of the blood to extract DMTS since we hypnotized plasma portion has more partitioned DMTS than blood cells portion due to the ability of plasma protein – DMTS interactions. Besides, plasma is less complicated than cells in terms of chemical composition as well as complexity of development of an extraction method. The DMTS extraction protocol employed for the plasma was very similar to the extraction protocol employed for cells except the lack of the sonication step since there is no need to lyse cells. However, it is observed that appoximately 4.5 times higher peak for DMTS is obtained for the cells portion than in the plasma portion for a same blood sample. The blood sample was taken from a rat 3 hours after intravenously injecting a DMTS dose of 10 mg per 1 mL of blood. Figure 11. shows DMTS peaks obtained for plasma and cells portions of this blood sample showing DMTS is partitioning more to the cells portion of a blood sample. The partitioning of DMTS between cyclohexanone (organic later) and 15% w/w Poly80 (aqueous layer) was studied and the results are shown in Figure 11. DMTS partition coefficient was approximately 3 – 3.5.

Conclusions about analytical method development for measuring DMTS in blood

A preliminary HPLC-UV absorbance method for determining the concentration of DMTS in blood has been developed. The method is based on DMTS extraction into cyclohexanone. The range of reliable quantitation for rat blood was found to be 0.04 – 0.30 mg/mL, that includes the concentration range when DMTS is applied in the maximum tolerable doses (20 mg/kg Intravenous, 200 mg/kg intramuscularly) for pharmacokinetics studies -will be published later. The experiment in blood was repeated with new blood samples three months after the original experiment, and excellent reproducibility was obtained. When plasma or whole blood was analyzed, we did not get measurable results. However, from RBCs a low (about 7%), but reproducible recovery was achieved. This is likely due to a number of factors. It may be because the majority of the DMTS was bound to serum albumin, and that was either discarded with the plasma and/or not extracted. Future experiments will test this hypothesis. Due to the matrix effects that were observed samples measured via this method should be spiked with a small amount of DMTS to provide a measure of the sensitivity. When this analytical method was employed for preliminary pharmacokinetic experiments (see below in chapter II.1.3.2), it was proved to be successful for measuring the DMTS levels in the samples, however, it is recommended to optimize the sample preparation method to decrease the blood sample volume for the analysis. We are planning to use Solid Phase Micro-Extraction (SPME) / GC-Headspace measurement directly from Blood (as we previously used for the micelles: Chapter II.1.1.1).

II.1.3.2. Absorption Kinetics and Residence Time determination for DMTS in rat model

II.1.3.2.1. Absorption kinetics: Three rats were given 200 mg/kg DMTS (50 mg/ml DMTS-15%Poly80), and blood samples were taken at periodic time intervals, and tested for DMTS by HPLC (see method in section II.1.3.1.). The results indicated that the absorption of DMTS occurs very quickly, (in about 5-15 mins) as shown on Figures12a and 12b. This means that the Poly80 formulated DMTS offers immediate protection when injected intramuscularly. For applying standardized higher DMTS dose, further development of advanced formulations is necessary.

II.1.3.2.2. Residence time: For the residence time determination Poly80 formulated DMTS (50 mg/ml-15%Poly80) was injected intravenously into rats at the dose of 20 mg/ml, and blood samples were taken at periodical time intervals. Blood samples were analyzed as described in section II.1.3.1. These preliminary results are shown on Figure 13, indicating the estimated half life of 36 mins. This circulation time allows to provide protection for a reasonable time interval.

II.1.4.1. Formulation Development for DMTS and Cbi combination

Analytical Determination of DMTS and Cbi combination

Since Cbi is not volatile, it cannot be measured by GC. We developed HPLC method for measuring Cbi and DMTS in the combination. Figure 14a shows the calibration curve for DMTS alone (GC-MS Method), Figure 14b shows the calibration curve for DMTS alone (HPLC-UV method). Figure 15. shows the calibration curve for DMTS in the

mixture of DMTS-Cbi when DMTS:Cbi = 1:2.5 (m/m). Figure 16. shows a representative chromatogram for DMTS-Cbi mixture by the HPLC method. These methods were successfully used for the *in vitro* stability studies (Figures 17a and 17b) for the DMTS-Cbi combinations, however, for further PK studies, HPLC-MS-MS methods are planned to get exposed (either at SDSU by Dr. Logue, or here at SHSU, Forensic Department by Dr. You).

Formulation Development for (DMTS+Cbi 15% Poly80) Combination

The detailed formulation is described in the experimental section (Appendix 2-10).

II.1.4.2. *In vitro* Stability Studies with DMTS and Cbi combination

The previously described analytical method was employed for determining the *in vitro* stability for the mixtures of DMTS-Cbi. The stability studies were carried out accordingly to the protocol described in II.1.2 (DMTS alone) and Appendix 2-11. Figure 17a and 17b shows the results of the stability studies.

At +4 ^{0}C temperature, both the Formulation A and Formulation B samples show stability close to 100%. At room temperature the formulation A samples still showed reasonable stability, but in the Formulation B samples the stability decreased with time. The biggest loss was noted at the heated samples at +40 ^{0}C. The sample analysis, and the possible degradation product identification is under investigation now. Analysing the samples by GC-MS, we found a degradation products of dimethyl-disulfide and dimethyl-tetrasulfide, that is in agreement of the earlier report by Chubachi (Chubachi et al, 1966), that higher temperature a chemical disproportion can occur. We did not find this degradation product at the heated samples when there was no Cbi present in the

system. However, based on the thermodynamical approach of chemical reactions, if a reaction is happening at a higher temperature within a short time, it does not mean that it will happen at lower temperature at longer period of time. A reaction does not proceed without reaching the activation energy that can be provided by heating. (It needs to be noted, that each time there was an initial loss when compared the first sealed sample to the original sample before pipetting to the container (Picture1). Since the DMTS concentration is not degreasing significantly over 4 weeks at the temperature of +4 ^{0}C, if the time zero would have been measured the same way of opening a sealed container, it would show a close of 100% remaining at +4 ^{0}C for 4 weeks).

Based on these data, the double sealed refrigerated samples are declared to be stable (close to 100% recovery) over 4 weeks.

II.2. Year 01 Milestone #2: Demonstrate initial *in vivo* efficacy with DMTS alone and in combination in the chosen formulations (micelles, lipid emulsion). *Success*: Mortality is reduced by 50% after at least a 2xLD_{50} CN challenge followed with IM administration of antidote(s). *Rationale:* By developing a more efficacious, IM administered CN antidote, our preparedness for successfully treating victims in a mass casualty situation will be enhanced. (Specific Aims 1 and 2)

☐ Met ☐ Not Met ☒ In Progress

II.2.1. *In vivo* Efficacy for DMTS alone

Therapeutic in vivo experiments with micellar DMTS formulations (mDMTS)

Based on the solubility studies (Figure 1), a preparation of 26.7 mM PEG_{2000}-DSPE + 2.5 mg/ml of DMTS was selected for *in vivo testing*. Results proved that DMTS is an effective antidote in antagonizing CN intoxication, since an antidotal protection of 2xLD50 was seen at the low dose dose of 12.5 mg/kg (Table 1). It is believed that similarly to the *in vitro* result, the *in vivo* efficacy would increase with the applied dose, but the present formulation (mDMTS) would not allow for a higher dose because the injection volume would not be tolerated by the mice. Comparing the efficacy of DMTS with that of earlier tests performed with TS and methyl propyl trisulfide when TS at doses of 100 mg/kg and 200 mg/kg provided APRs of 1.1 and 1.25 respectively, and methyl propyl trisulfide at doses of 100 mg/kg and 200 mg/kg provided APRs of 1.2 and 1.67 respectively it can be concluded, that DMTS is significantly more effective than both investigated sulfur donors (TS and methyl propyl trisulfide), because a higher APR of 2 was reached at a much lower DMTS dose of 12.5 mg/kg (Kovacs et al, 2013). These test also proved that the intramuscular route of administration is effective in case of mDMTS, because the effect of the antidote was immediately showing that the absorption of the antidote candidate is rapid enough to counteract the fast acting CN.

In Vivo Efficacy Studies with Poly80-Formulated DMTS (15% Poly80-DMTS)

The formulation studies resulted in compositions that are capable of solubilizing DMTS in concentrations high enough to be tested in mice, thus animal studies were performed with the developed compositions. DMTS was then tested *in vivo* using Poly 80 as the solvent system for the antidote. This allowed for a higher dose to be evaluated and the

drawback of the low DMTS concentration associated with the micelle preparation was also eliminated, thus injection volumes were substantially decreased. Table 2 shows the therapeutic antidotal protections, expressed as Antidotal Potency Ratio (APR), with DMTS in various formulations and applied doses: 50mg/mL DMTS in 15% polysorbate 80 at doses of 50, 100 and 200 mg/kg and 50 mg/ml DMTS in 20% Poly80 at the dose of 100 mg/kg. The APR values for TS at the doses of 100 and 200 mg/kg are also shown for the purposes of comparison. DMTS showed remarkable *in vivo* antidotal protection at all the applied doses. The magnitude of the protection was directly proportional to the applied DMTS doses. At the dose of 100 mg/kg, DMTS showed about 3 times higher protection vs TS. This ratio is even higher 4.1/1.3 = 3.2 when the applied doses of the antidotes were 200 mg/kg. When DMTS doses higher than 200 mg/kg were applied intramuscularly, in the legs of animals significant muscle damage were noted, therefore with the mice model, it was not recommended to increase the DMTS dose over 200 mg/kg. The data presented in this study confirm the applicability of DMTS in the detoxification of CN. The APR of 4.1 with the DMTS in 15% Poly80 at the doses of 200 mg/kg represents the highest antidotal protection presently available for combating CN intoxication by a single antidotal molecule without combination.

In Vivo Efficacy Studies with 20% Poly80-DMTS

There was no significant difference in the antidotal protection when the 15% Poly80 was compared to the 20% Poly80 (Table 2). However, the APR was highly DMTS dose dependent. When the 20%Poly80 DMTS was prepared in two ways A) DMTS was added to the 20% Poly80 solution on the same day when the Poly80nwas prepared; B) DMTS was added on the next day (Table3). In both cases the solutions were clear without any opalicity or precipitation (Picture 2).

II.2.2. *In Vivo* Efficacy Studies with DMTS-Cbi Combination.

For the *in vivo* experiments, the combination formulation was prepared accordingly the Method A: First the 15% Poly80 DMTS (50 mg/ml) was prepared, and the required amount of Cbi was added as a powder to the solution. After vigorous vortexing, the cocktail was injected IM into the upper part of the mice.

The *in vivo* efficacy studies showed significant increase when DMTS was combined with Cbi. When 100 mg/kg DMTS dose was applied alone, the therapeutic antidotal protection was over three times LD50 (APR =3.1); and when 250 mg/kg dose of Cbi was applied alone, the therapeutic antidotal protection was about two and half times LD50 (APR =2.4). However, when DMTS and Cbi was employed in a combination (100 mg/kg DMTS dose and 250 mg/kg Cbi dose), the therapeutic antidotal protection was enhanced to almost six times LD50 (APR=5.9).

Section III. Year 01 Deliverables:

Deliverable 1. Reporting/publish results regarding antidotal efficacy studies, kinetic studies, and improved formulation/characterization and delivery of the investigated potential therapeutic agents against cyanide.

Peer-Reviewed Articles (2012-2013)

(1) Kovacs K., Ancha, M., Jane M., Lee S., Angalakurthi, S., Negrito, M., Rasheed S. Nwaneri, A., Petrikovics I. Identification, Solubility enhancement and in vivo testing of a cyanide antidote candidate. *European Journal of Pharm. Sci*. 49, 352-358. **2013.** (http://dx.dorg/10.1016/j.ejps.2013.04.007)

(2) Manage, B.W.M., Petrikovics, I. Confidence Limit Calculation Method for Antidotal Potency Ratios (APR) Derived from two LD_{50} Values Determined by the Dixon Method. *Journal of World Methodology,* 26, 3(1): 7-10, **2013**. (http://dx.doi.org/10.4329/wjm.v3.il.00)

(3) Yu, J.C.C., Martin, S., Nasr, J., Stafford, K., Thompson, D.E., Petrikovics, I. LC-MS/MS Analysis of 2-Aminothiazoline-4-Carboxylic Acid as a Forensic Biomarker for Cyanide Poisoning. *Journal of World Methodology*, 26, 2(5),1-7, **2012**. Doi:10.4329/wjm.v2.i5.1

(4) Bhandari, R.K., Oda, R.P, Youso, S.I, Petrikovics, I., Bebarta V.S., Rockwood, G.A. and Logue, B.A. Simultaneous determination of cyanide and thiocyanate in plasma by chemical ionization gas chromatography mass-spectrometry (CI-GC-MS). *Analytical and Bioanalytical Chemistry,* 404(8), 2287-2294, **2012** (http://dx.doi.org/10.1007/s00216-012-6360-5).

(5) Petrikovics, I., Yu, J.C.C., Thompson, D.E., Jayanna, P., Logue, B.A., Nasr, J., Bhandari, R.K., Baskin, S.I., Rockwood G.A. Plasma Persistence of 2-Aminothiazoline-4-Carboxylic Acid in Rat System Determined by Liquid Chromatography Tandem Spectrometry. *J. Chromatography B,* 81-84, 891-892, **2012**. DOI:10.16/j.jchromb.201201.024.

Peer-Reviewed Articles in preparation:

1) Bhandari, R.K., Oda, R.P., Petrikovics, I., Thompson, D.E., Brenner, M., Mohan, S.B., Bebarta, V. S., Rockwood, G.A. Logue, B. A. Cyanide Toxicokinetics: The Behavior of Cyanide, Thiocyanate and 2-Amino-2-thiazoline-4-carboxylic Acid in Multiple Animal Models..To be submitted to *Journal of Toxicokinetics*

2) Kovacs, K., Jayanna, P.K, Duke, A., Winner, B, Negrito, M., Angalakurthi, S., Yu,J. C.C., Füredi,P., Ludányi,K., Rockwood, G.A. and Petrikovics, I. Micellar Encapsulation of a Novel Sulfur Donor for Cyanide Antagonism. To be submitted to *Drug Development and Industrial Pharmacy (Informa Healthcare Journals)*

3) Kovacs, K., Duke, A., Shifflet, M., John C., Winner, B., Nwaneri, A., Lee, S., Olvera, J., Rockwood, G.A. and Petrikovics, I. Parenteral dosage form development and testing of dimethyl trisulfide, as an antidote candidate to combat cyanide intoxication. To be submitted *to Pharmaceutical Development and Technology (Informa Healthcare Journals)*

4) Duke, A., Lee, S., Jane, M., Aleman, J., Fisher, D., Barcza, T., Kovacs, K., Rockwood, G.A. and Petrikovics, I. Stability as a function of temperature, pH and time of a polysorbate80-formulated dimethyl trisulfide, as a cyanide antidote candidate. To be submitted to *Pharmaceutical Development and Technology (Informa Healthcare Journals)*

5) Petrikovics, I., Budai. M., Kovacs, K. and Thompson, D.E.: Past, Presence and Future of Cyanide Antagonism Research (From the ancient remedies to the recent combination therapy). Announced to be published in *World J. Methodol, WJM, online ISSN 222-0682, DOI:10.5662, ID#: 02446322*

Bookchapter:

1) David Thompson and Ilona Petrikovics. Cyanide physicochemical properties, synthesis, uses and applications in: "Toxicology of Cyanides and Cyanogens: Experimental, Applied and Clinical Aspects", and will be published by John Wiley & Sons, Chichester, UK.
(Submitted in December**, 2012**)

Peer-Reviewed Presentations/Posters (2012-2013)

(1) Brenner, M., Mahon S., Boss, G., Lee, J., Petrikovics, I., Patterson, S., Rockwood, G.A Collaboration Studies for Acceleration of advanced CN Antidote Agents for Mass Casualty Exposure Treatments: DMTS. *NIH CounterAct Meeting, Bethesda, MD., June 25-27, 2013 (C.4. Page 96).*

(2) Rockwood, G., Petrikovics, I., Logue, B., Boss, G., Mahon, S., *In vivo* efficacy and optimization of novel cyanide countermeasures [IAA AOD 12060-001-0000/A 120-B.P2012-01) *NIH CounterAct Meeting,* June,25-27, Bethesda, MD , **2013** (C.3. Page 96).

(3) Negrito, M., Kovacs, K., Ancha, M., Jane, M., Lee, S., Angalakurthi, S., Rasheed, S., Petrikovics, I.* Solubility Enhancement Studies for a Potential Cyanide Antidote. 52th Annual Meeting of the Society of Toxicology, March 10-14, **2013**, San Antonio, Texas. (Abstract #: 2054, Poster #: 111, Page #: 301)

(4) Petrikovics, I*., Kovacs, K., Budai, M., Winner, B., Negrito, M., Jayanna, P., Furedi, P., Rockwood, G. Lipid based formulations for pre-clinical application of therapeutic agents against cyanide intoxication. World Congress of Clinical Lipidology, December, **2012**, Budapest, Hungary.

Presentations at Regional Meetings

(1) Angalakurthi, S.K*, Coveyou, K., Kovacs, K., Petrikovics, I. Reversed-Phase HPLC Method Development for a Cyanide Antagonist Candidate, *ACS Regional Meeting,* November, **2012***,* Baton Rouge, TX.

(2) Duke, A., Rasheed, S*., Kovacs, K., Petrikovics, I. Solubility Enhancement for Poorly Water Soluble Drugs, *ACS Regional Meeting,* November, **2012***,* Baton Rouge, TX.

(3) Negrito, M*., Winner, B., Rasheed, S., Kovacs, K., Petrikovics, I. Optimization of Micellar and Emulsion Type Formulations for Developing Cyanide Antidotes, *ACS Regional Meeting,* November, **2012***,* Baton Rouge, TX.

Deliverable 2. Continue tracing and reporting results of *in vivo* comparison studies across established and candidate cyanide countermeasures.

Deliverable 3. Continue participation in collaborations with other research groups nationwide on the project. Publishing/reporting results and ongoing information exchange with the other research groups.

Coordination with other organizations conducting related work (Dr. Sari Mohan, Beckman Laser Institute and Medical Clinic, UC-Irvine, Irvine, CA 92612. Rabbit blood and plasma samples have been sent to SDSU after exposure to cyanide in a number of types of experiments).

Section IV. Summary of Research Results

IV.1. Key Research Accomplishment

Studies for DMTS alone:

- Formulation development and optimization for IM injection
  - We developed formulations with micelles, co-solvents, surfactants
    - Polysorbate 80 (15% and 20%) were chosen for further studies

- Analytical method development
  - We developed method to measure DMTS in
    - mDMTS (SPME-GC-MS)
    - DMTS –Poly80 (GC-MS and HPLC-UV)
    - Measuring DMTS in blood (Liquid-liquid ample preparation method development) (HPLC-UV)

- Stability studies with 15% Poly80-DMTS and 20% Poly80-DMTS
  - We checked the *in vitro* stability at various storage conditions at various pH-, and temperatures over a month (Figures 7a-e).
  - We established a storage condition (pictures 1 and 2), at which we could prevent the evaporation/degradation, and at $+4^0$C and pH=7, the DMTS showed stability over 95% during the study of one month.

- *In vivo* efficacy studies
  - The therapeutic antidotal efficacy was characterized by antidotal Potency Ratios (APR= LD50 of CN in the presence of the antidote/LD50 of CN without antidote) (Mice model)

    - mDMTS, (APR=2.1; DMTS Dose=12.5 mg/kg)

    - 15% Poly80-DMTS (APR= 3.9; DMTS Dose=100 mg/kg)
      (APR= 5.4; DMTS Dose=200 mg/kg)

    - 20% Poly80-DMTS (APR=3.0-3.25; DMTS Dose=100 mg/kg)

- Pharmacokinetics (Rat model)
  - Residence time determination (t 1/2 = 36 mins)
  - Absorption kinetics (Tmax = 5-15 mins)

Studies for DMTS+ Cbi combination:

- Formulation development (15% Poly80-DMTS+Cbi)
- Analytical method development (HPLC; Spectrophotometry)

- Stability studies with 15% Poly80-(DMTS+Cbi) (In double sealed containers, $+4^0$C temperature over 16 days) (pictures 1 and 2)
- *In vivo* efficacy studies(15% Poly80-DMTS+Cbi) (mice model)
  APR= 5.9 (100 mg/kg DMTS+250 mg/kg Cbi)
  APR=2.4 (250mg/kg Cbi)
  APR=3.9 (100 mg/kg DMTS)

IV.2. Conclusion (Summary of the results)

Studies with DMTS alone:

The first formulation attempt focused on micellar encapsulated DMTS (mDMTS) preparation. This was a suitable formulation for IM injection, however, the maximum DMTS load was 2.5 mg/ml, (Figure 1) what could provide only a DMTS dose of 12.5 mg/kg. Since the antidotal protection is directly proportional to the dose and the 12.5 mg/kg DMTS dose provided the maximum APR of 2.1, (Table 1), the choice was the following: we either need to increase the injection volume of mDMTS, or find a better excipient to achieve a higher DMTS concentration (achieving higher dose, while the injection volume is still within the required range).

Searching further with the solubility studies, we tried a series of various co-solvents and surfactants and their combinations, and some cyclodextrin derivatives (Figures 2, 3, 4, 5). Polysorbate 80 (Poly80) was chosen for further in vivo efficacy and pharmacokinetics studies: It is an FDA approved excipient, used in industry for many drug formulation. With 15% Poly80, the maximum solubility of 85 mg/ml was achieved with DMTS. We also tried 20% Poly80, what provided a maximum solubility of 110 mg/ml. Considering other factors (e.g. keeping the excipient concentration in the body as low as possible), we focused

on the 15% Poly80 formulation, however we did some experiments with the 20% Poly80 (Figure 6) formulation too (stability studies and in vivo efficacy studies). The stability studies at various pH (2, 4, 7, 9, and 11), temperatures (+4 ^{0}C, +20 ^{0}C, +40 ^{0}C). We established a storage condition (double sealed container that prevents from evaporation and degradation, Picture 1 and 2), at pH=7 we could achieve a stability over 95% for the period of the study of one month (Figures 7a-e). When we compared the stability of 15% Poly80 vs. 20% Poly80, we did not see significant difference (Figure 8).

For the analysis of DMTS in various formulations, GC-MS and HPLC analytical methods have been developed (Figures 14a, 14b, Figures 9, 10, 11).

The in vivo efficacy studies were run on mice model. The therapeutic antidotal protection was expressed as Antidotal Potency Ratio (APR=LD50 of CN with antidote/LD50 of CN without antidote). The APR was increased with increasing DMTS doses (Table 1 and 2). There was no significant difference between APR with 15% Poly80-DMTS vs. 20% Poly80-DMTS. DMTS showed significantly higher in vivo efficacy vs. TS. This is also true for the in vitro efficacy of DMTS vs. TS (reported earlier).

The pharmacokinetics study with 15% Poly80-DMTS on rat provided kinetical parameters (residence time after in vivo administration: t1/2=36min; and absorption kinetics: Tmax:5-15 mins after intramuscular administration). These parameters indicated that the formulation of Poly80 provided rapid absorption, and the DMTS remains in the circulation for a reasonable time (Figures 12a and b; and 13).

Studies with DMTS+Cbi Combinations:

The combination formulation (50 mg/ml DMTS + 125 mg/ml Cbi) with 15% Poly80 have been tried in two different ways: A) the 15% Poly80 DMTS was prepared first, and the required Cbi was added in a powder form, B) both the DMTS and the Cbi was dissolved in the Poly80 solvent, and they were combined together.

Analysis: Since the Cbi is not volatile, the combination was only measured on HPLC (Figure 15a, b and 16). The stability studies with the combination showed, that the DMTS remained stable (over 95%) in double sealed container (Picture 2) during the 4 weeks study. The room temperature samples showed some degree of degradation, especially with sample series B, but the heated samples degraded dramatically in both Series A and B. The identification of the products are under investigation. The *in vivo* efficacy studies clearly indicating, that the DMTS+Cbi combination provide significantly better protection than each components alone (APR= 5.9 (100 mg/kg DMTS+250 mg/kg Cbi); APR=2.4 (250mg/kg Cbi); APR=3.9 (100 mg/kg DMTS).

IV.3. Future Directions, Recommendations

- **The Combination of DMTS-Cbi looks very promising**
- **Optimization of formulation is necessary**
- **Optimization of antidote's dose**
- **Future PK studies with the combination**
- **Analytical method development for the combination in blood**

Section V. Proposed Year 02 Milestone #1 Y2 Milestone1: Continue comparison of candidate cyanide countermeasures (including organosulfur-based, cobalamin-based, and, upon consultation with NIH, other classes of candidate compounds) against currently available, established cyanide countermeasures, such as sodium thiosulfate, sodium nitrite and hydroxocobalamin and candidate CN antidotes under development such as cobinamide and sulfanegen. Demonstrate *in vitro* and *in vivo* efficacy with at least one SD. *Success*: SD reactivity is at least 10X higher than with thiosulfate. *Rationale:* Currently, DMTS is a promising candidate countermeasure; however it is uncertain that DMTS will ultimately emerge as a fieldable CN countermeasure. Therefore, efforts will continue to evaluate/identify next generation CN antidotes. (Specific Aims 1,2)

Section VI. Literature Cited

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Kovacs K, Ancha, M, Jane M, Lee S, Angalakurthi S, Negrito M, Rasheed S. Nwaneri A, Petrikovics I. 2013: Identification, Solubility enhancement and in vivo testing of a cyanide antidote candidate. *European Journal of Pharm. Sci*. 49:352-358.

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Section VII. Changes in Research Support

No changes have occurred in research support over the current reporting period.

APPENDIX 1.

FIGURES, PICTURES AND TABLES

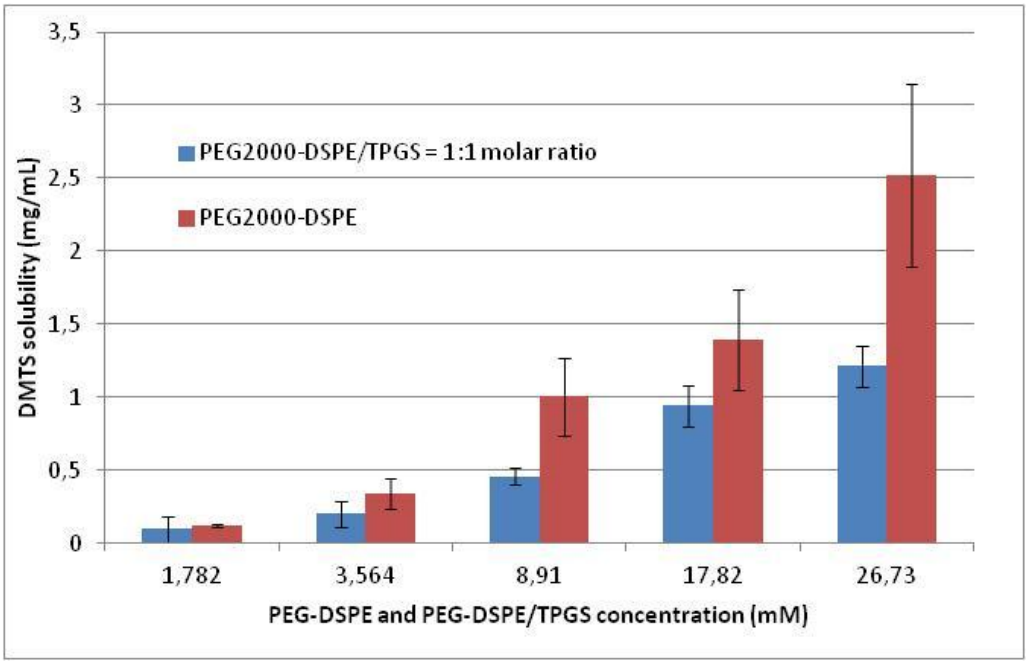


Figure 1. Solubility of DMTS in PEG_{2000}-DSPE micelles and mixed micelles comprising PEG_{2000}-DSPE/TPGS (molar ratio 1:1)

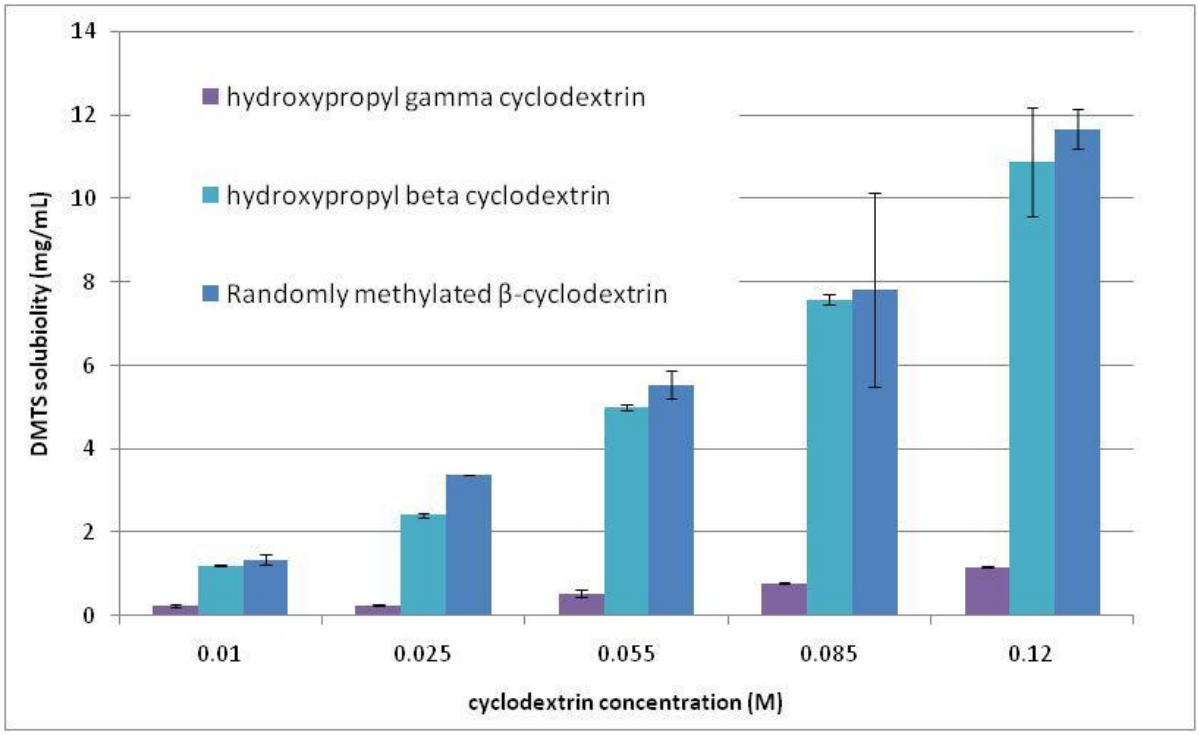


Figure 2. Solubility of DMTS in cyclodextrin solutions

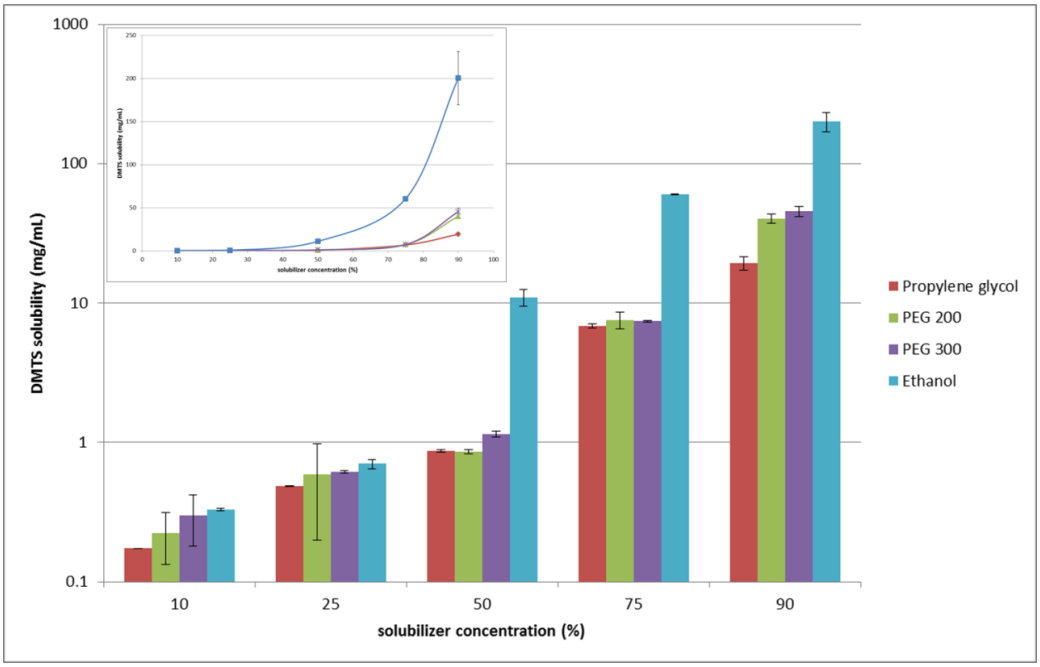


Figure 3. Solubility of DMTS in various co-solvents at increasing concentrations

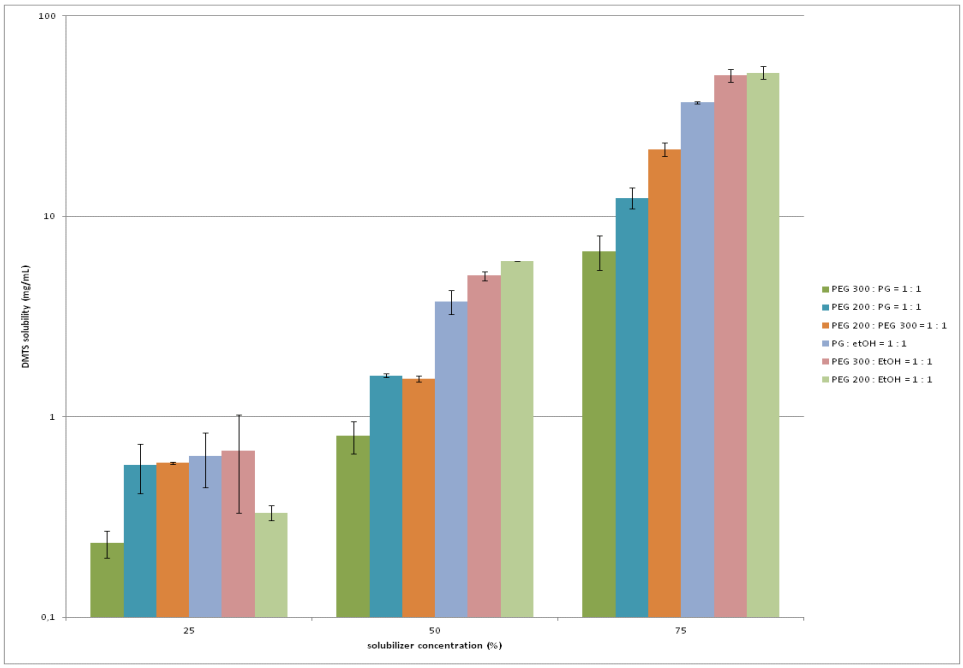


Figure 4. Solubility of DMTS in various co-solvent combinations at increasing concentrations

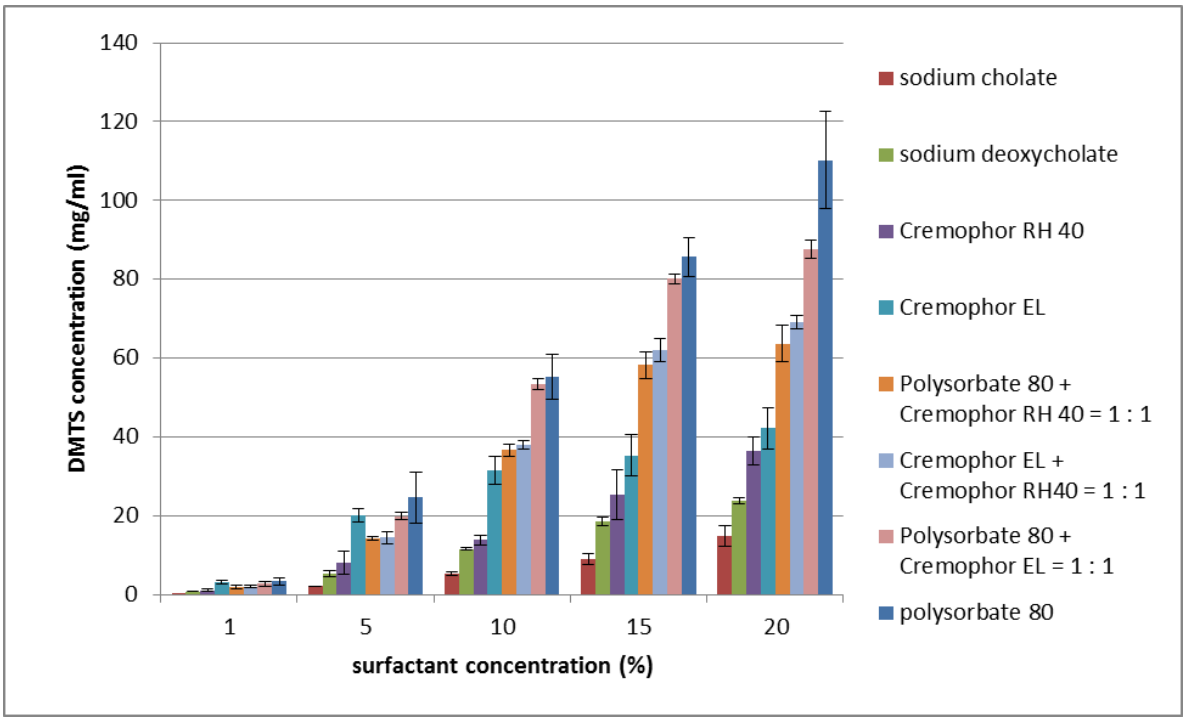


Figure 5. Solubility of DMTS in various surfactants at increasing concentrations

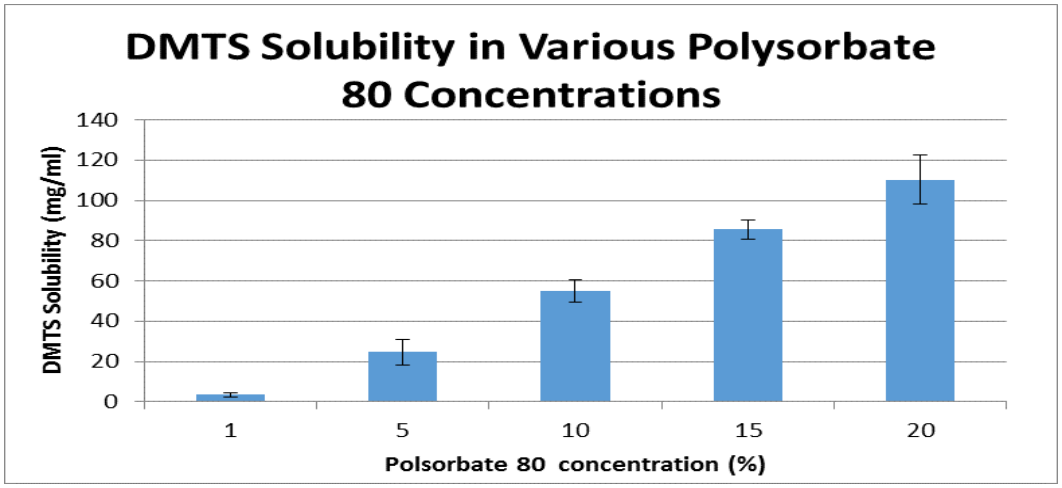
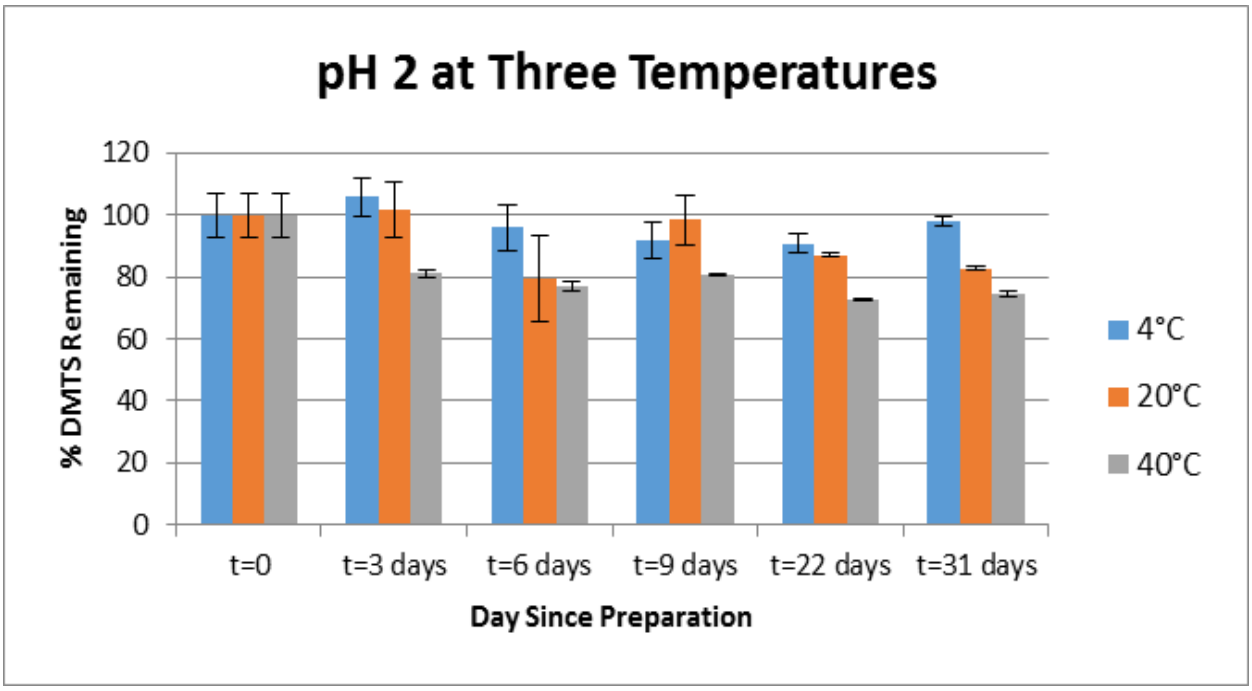
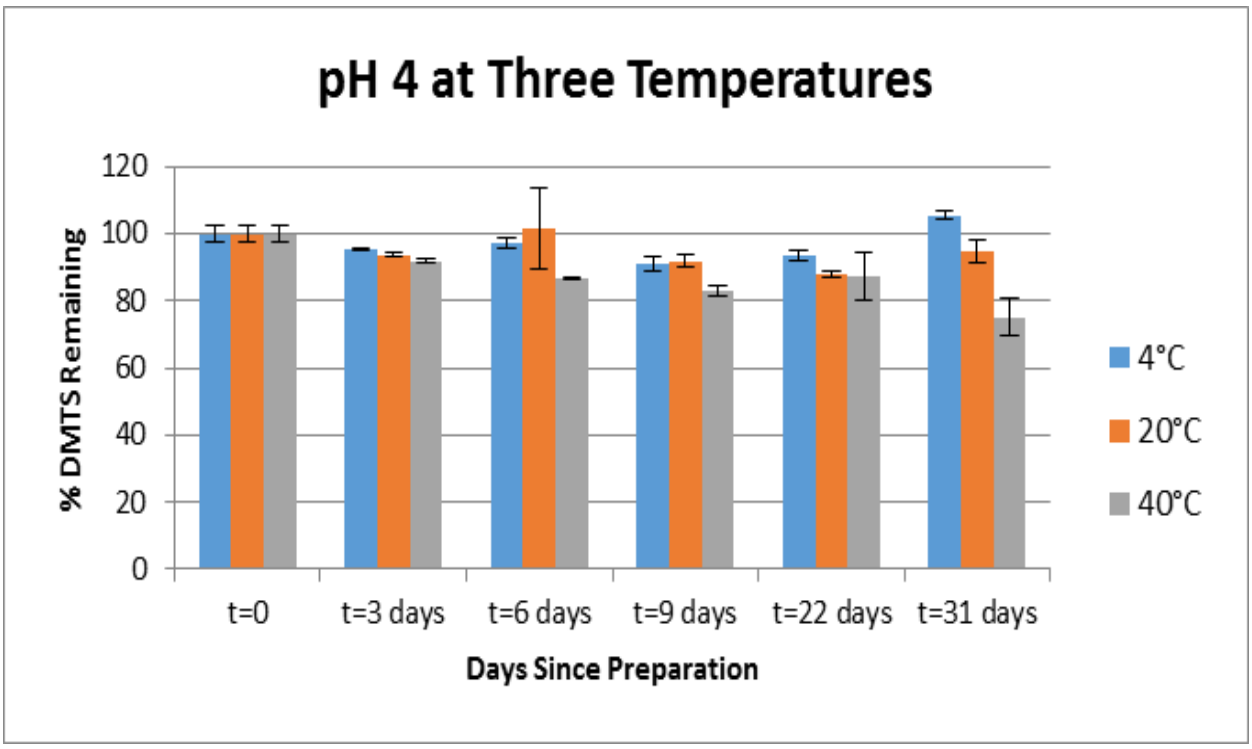


Figure 6. DMTS solubility in various concentrations of Poly80 solvent systems (Kovacs et al., 201


pH 2 at Three Temperatures
% DMTS Remaining
120
100
80
60
40
20
0
t=0
t=3 days
t=6 days
t=9 days
t=22 days
t=31 days
Day Since Preparation
4°C
20°C
40°C


pH 4 at Three Temperatures
% DMTS Remaining
120
100
80
60
40
20
0
t=0
t=3 days
t=6 days
t=9 days
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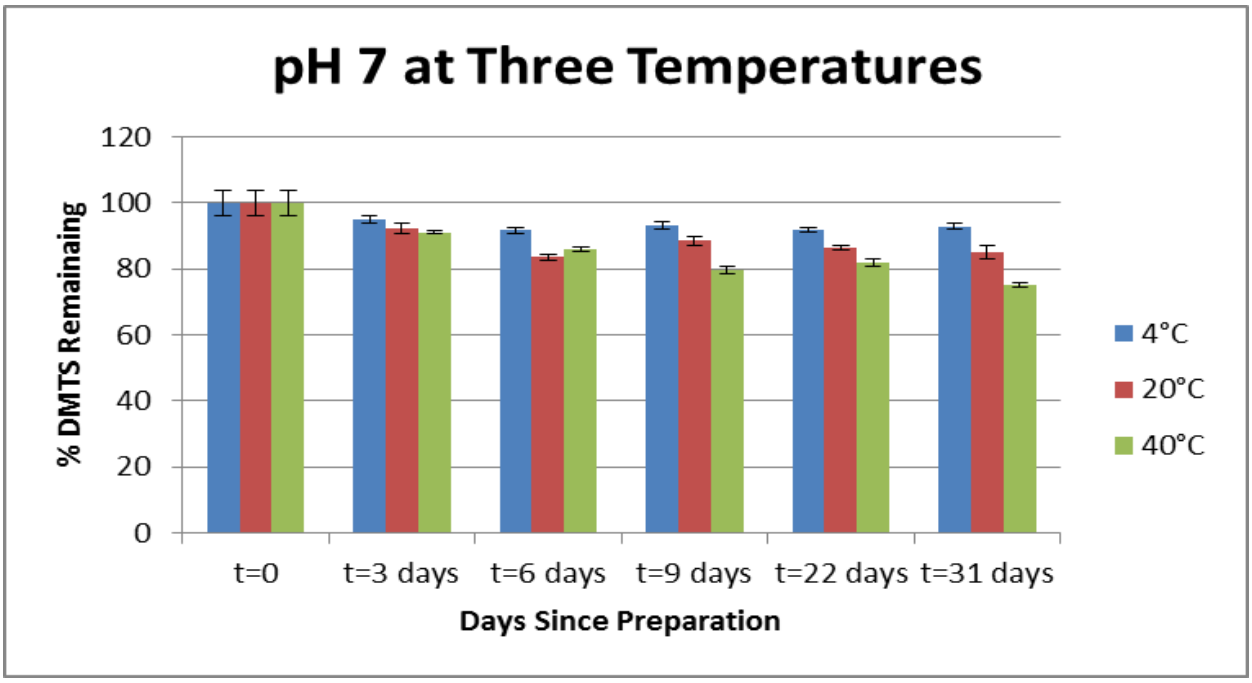
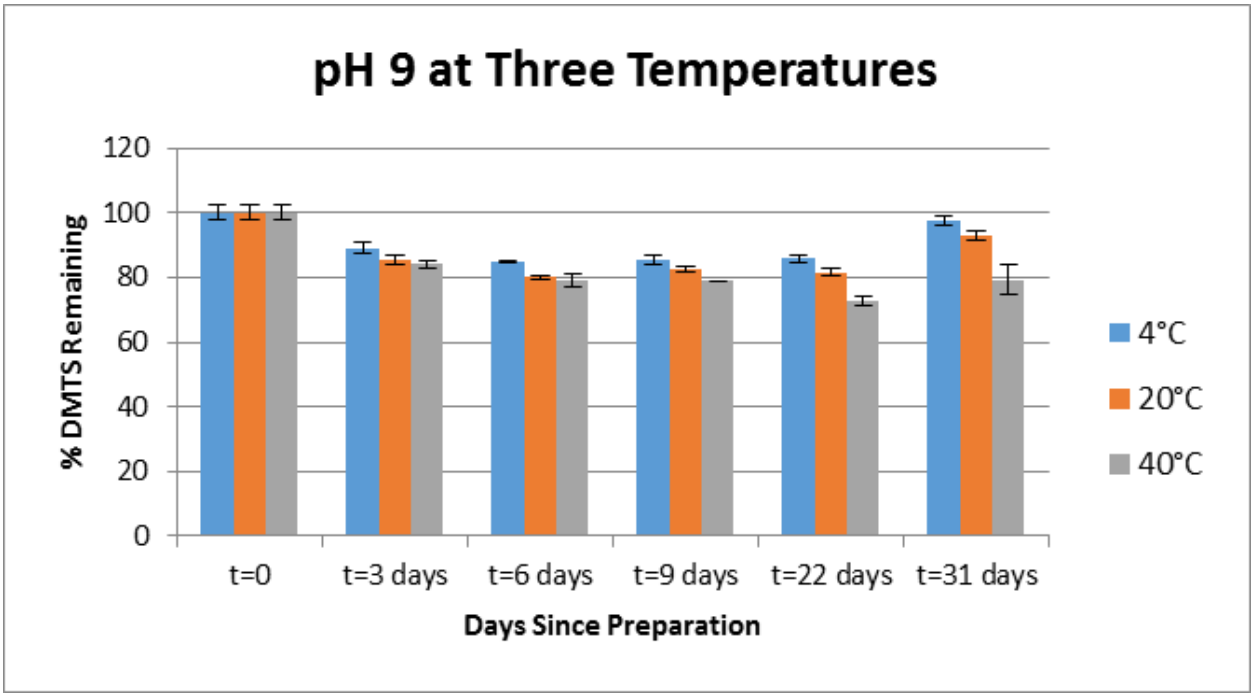
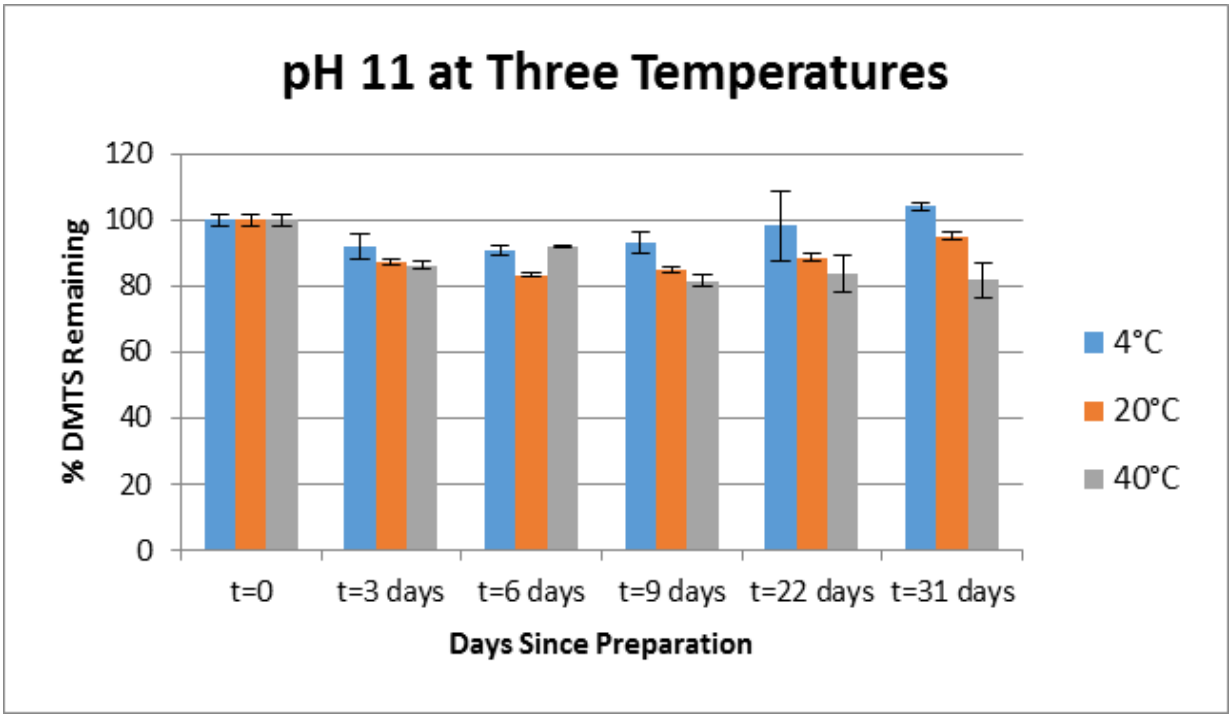
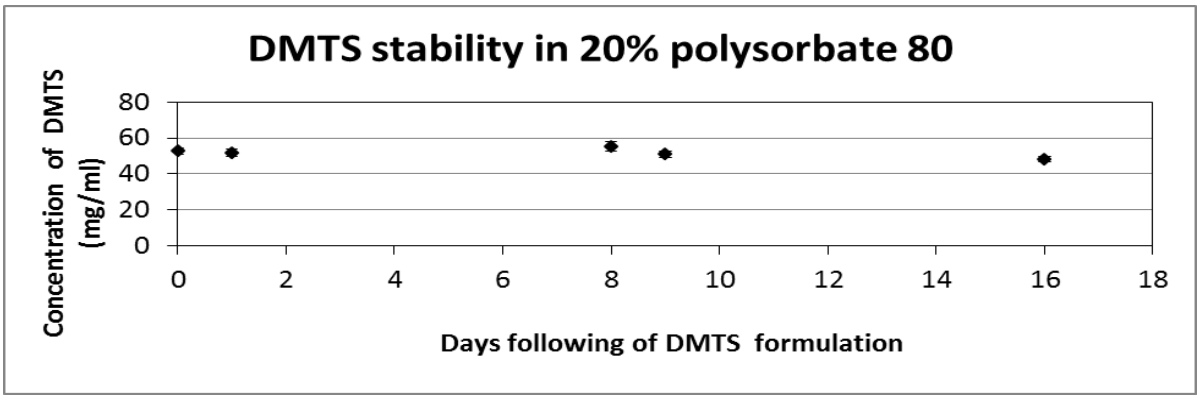
t=31 days
Days Since Preparation
4°C
20°C
40°C


pH 7 at Three Temperatures
% DMTS Remainaing
120
100
80
60
40
20
0
t=0
t=3 days
t=6 days
t=9 days
t=22 days
t=31 days
Days Since Preparation
4°C
20°C
40°C


pH 9 at Three Temperatures
% DMTS Remaining
120
100
80
60
40
20
0
t=0
t=3 days
t=6 days
t=9 days
t=22 days
t=31 days
Days Since Preparation
4°C
20°C
40°C



Figures 7a-e. DMTS Poly80 stability data, as a functions of time, temperature and pH. (note that as part of an improved preparation procedure, the DMTS formulation is now double sealed –Picture 1).



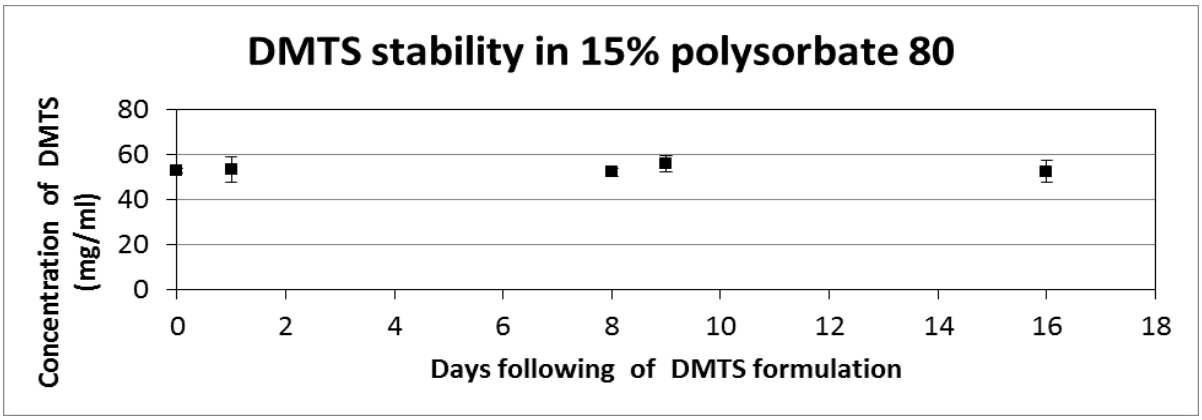


Figure 8. DMTS stability data for 15% and 20% Poly80-DMTS (50 mg/ml) measured by GC-MS

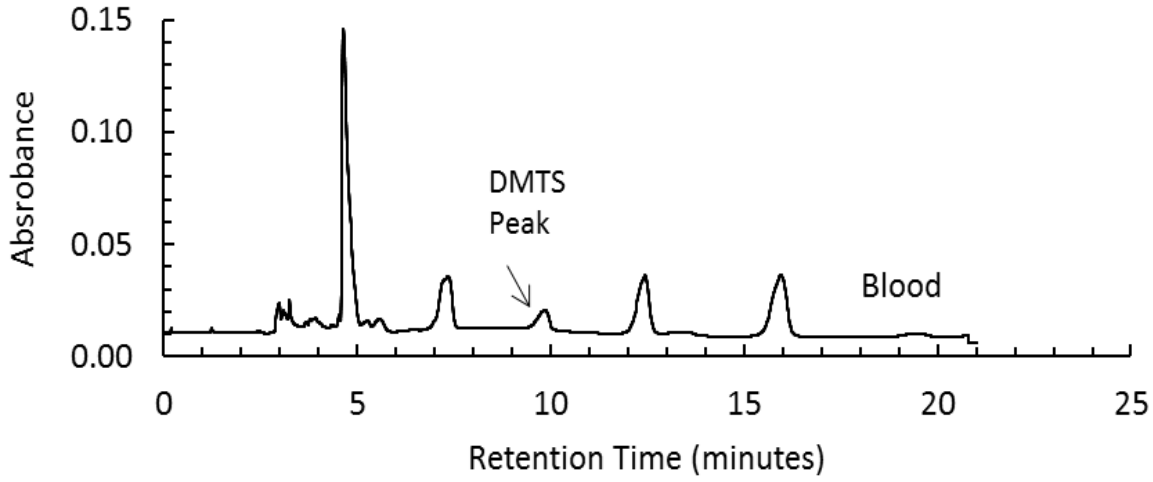


Figure 9. Typical HPLC Chromatogram for blood samples for DMTS Determination

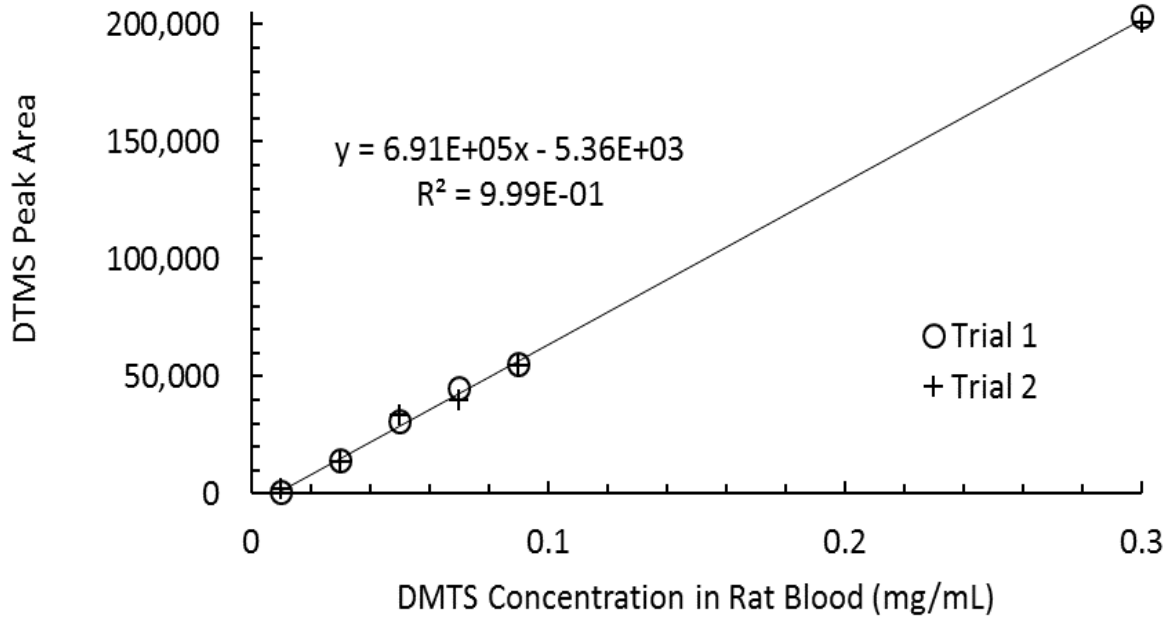


Figure 10. HPLC Calibration curve for DMTS in Rat Blood in the concentration range of 0.01-0.30 mg/mL

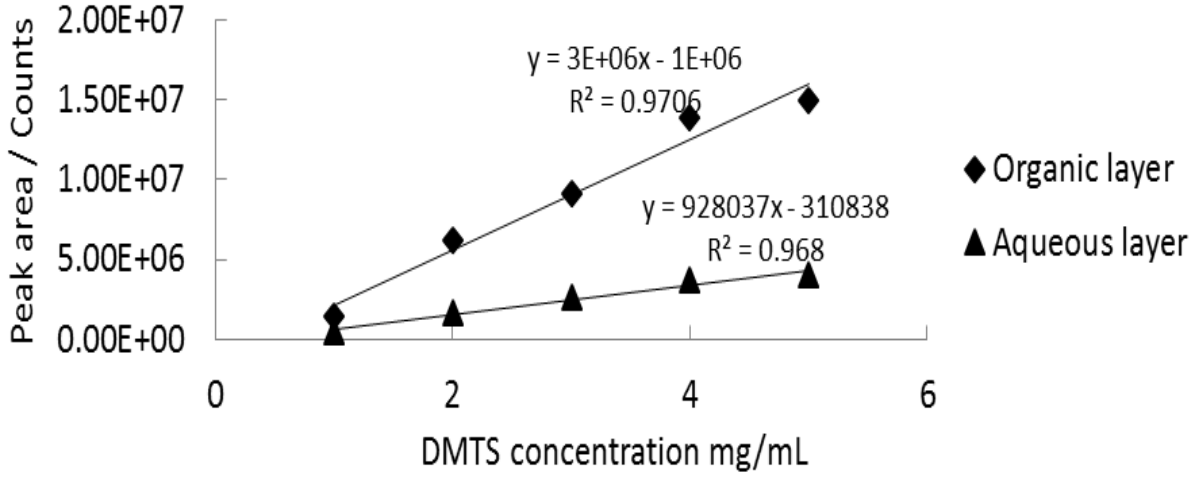


Figure 11. Partitioning of DMTS between cyclohexanone (organic layer) and 15% w/w Polysorbate 80 (aqueous layer) (HPLC-UV)

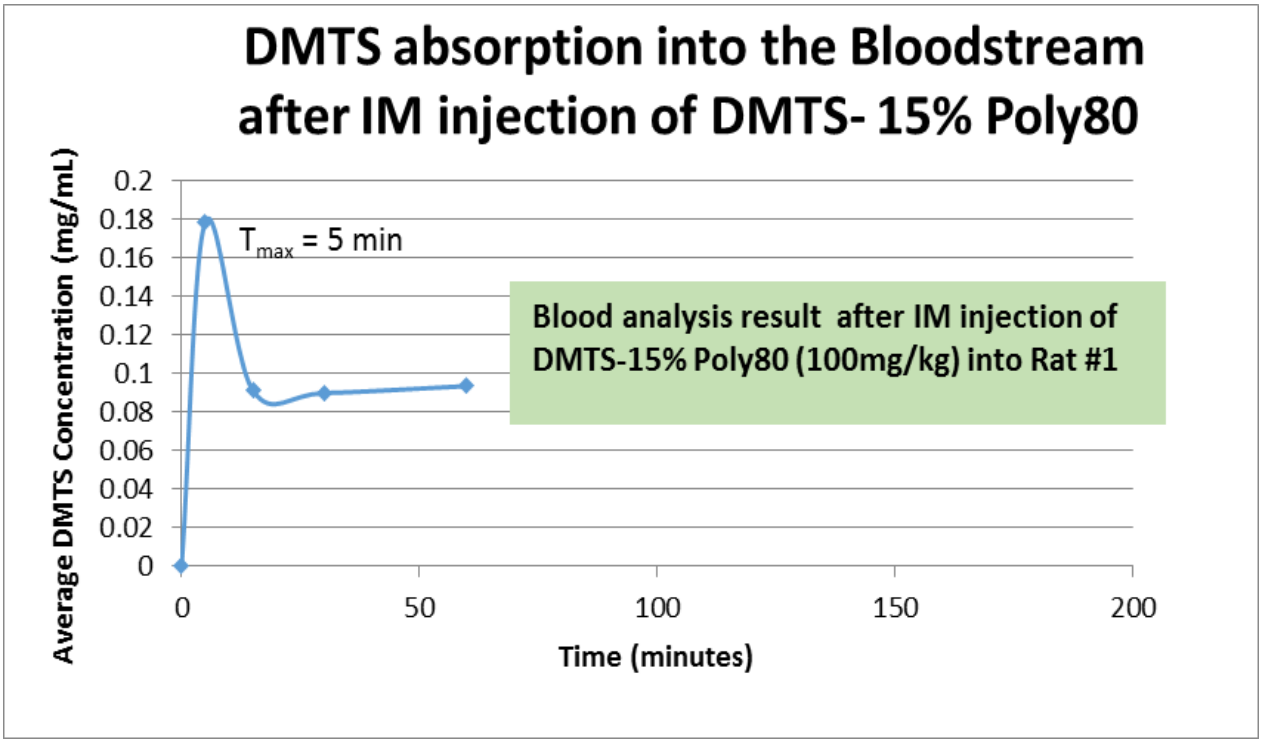


Figure 12a. Absorption kinetic curve when DMTS dose was 100 mg/kg (IM) (Measured by HPLC-UV)

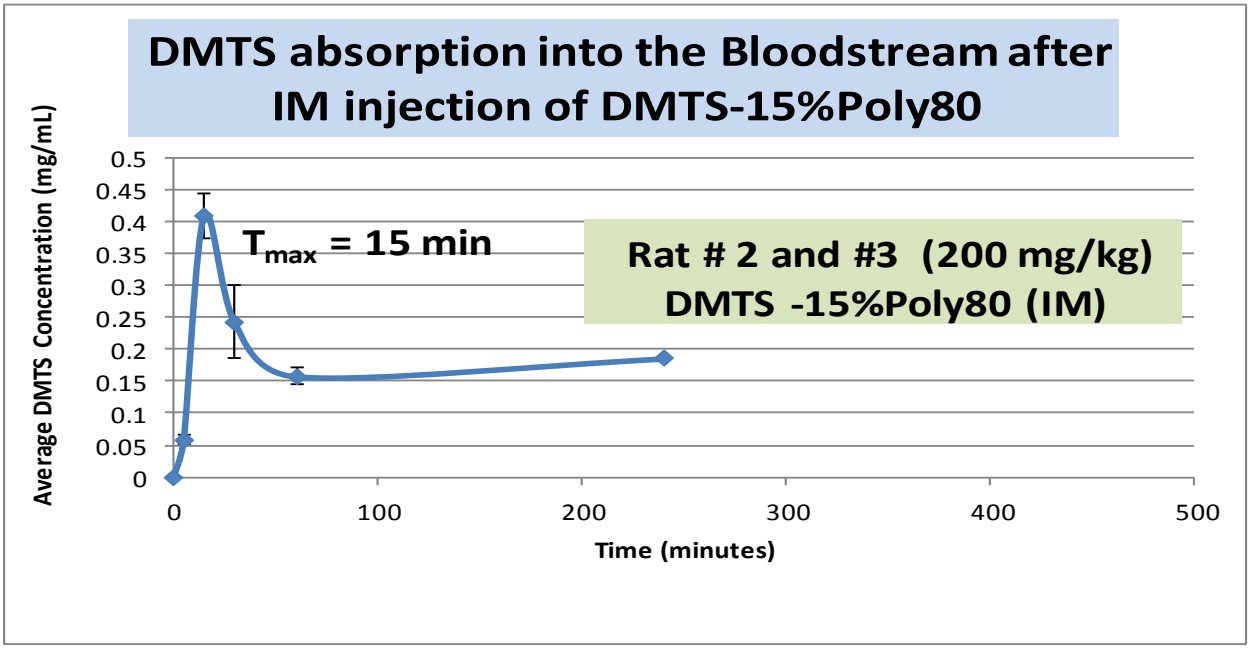


Figure 12b. Absorption kinetic curve when DMTS dose was 200 mg/kg (IM) (Measured by HPLC-UV)

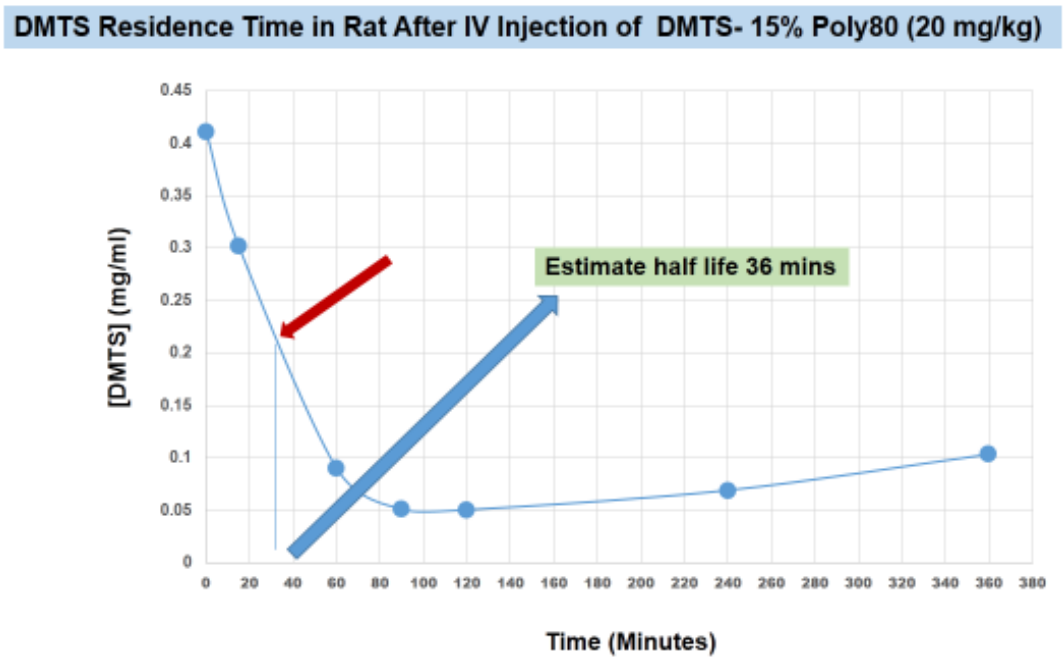


Figure 13. DMTS Residence time in rat after intravenous injection of DMTS-15% Poly80 (20 mg/kg)(Measured by HPLC-UV)

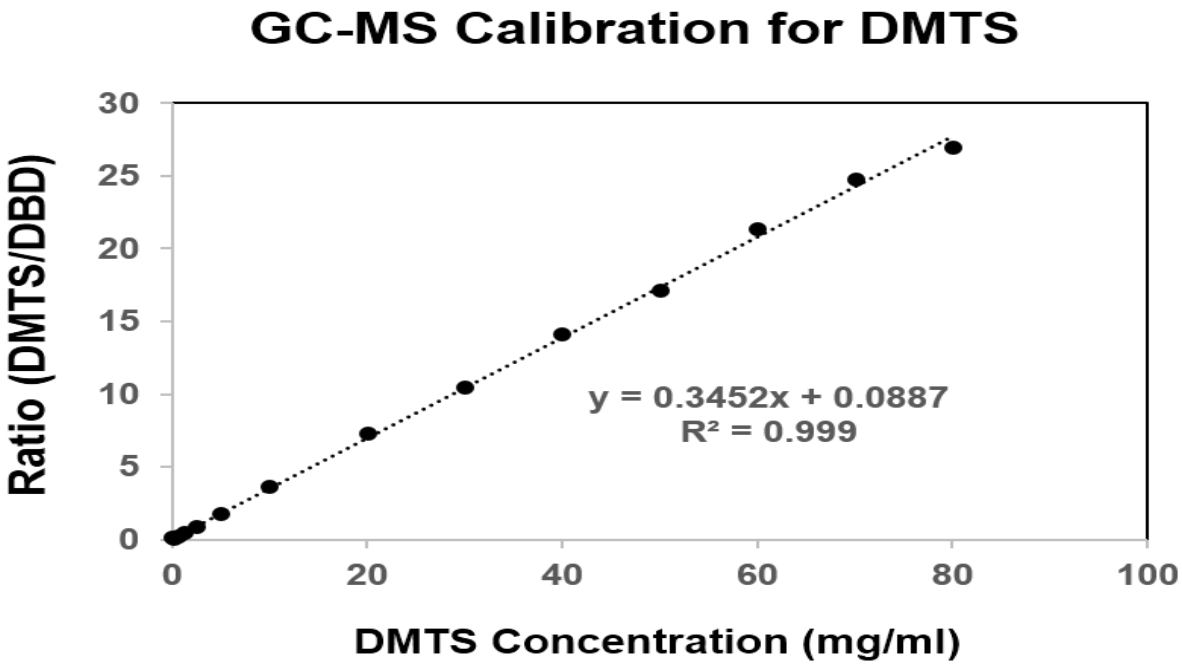


Figure 14a. Calibration curve for DMTS alone (GC-MS method)

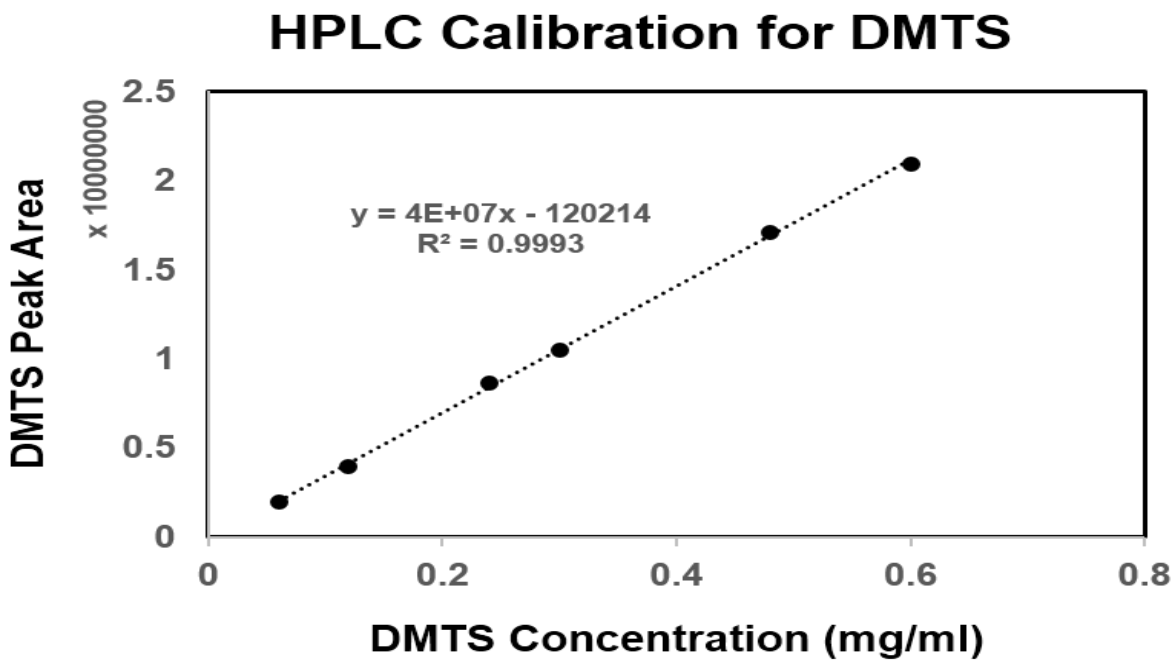


Figure 14b. Calibration curve for DMTS alone (HPLC-UV)

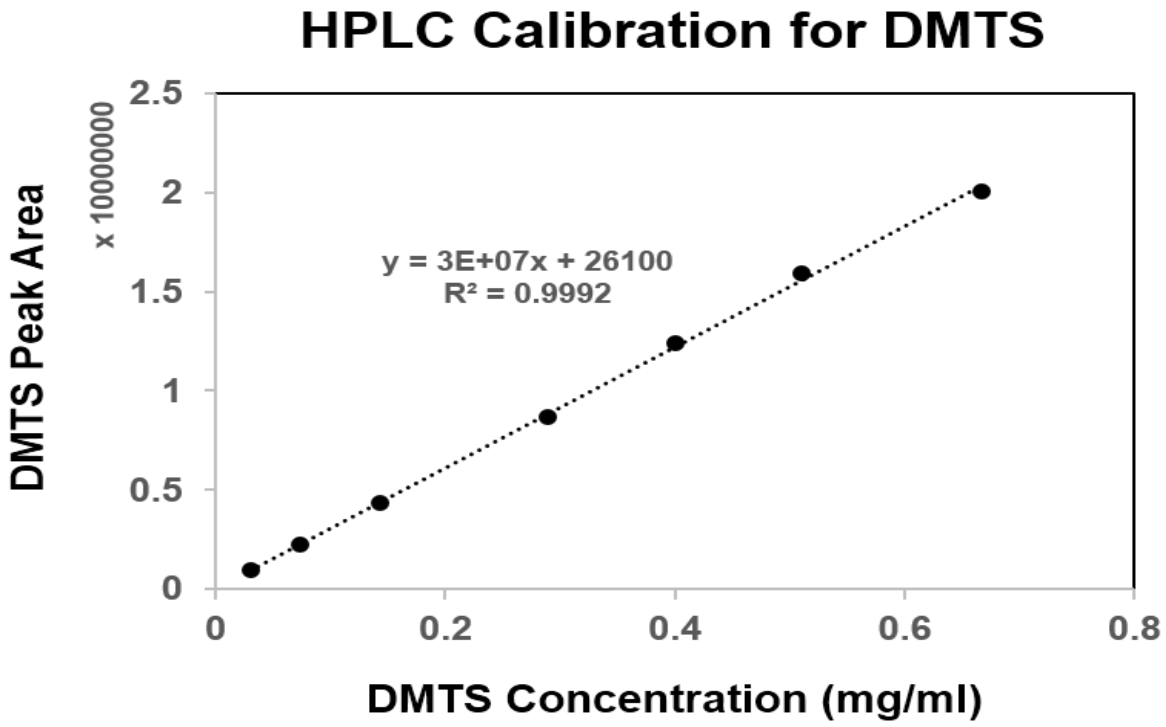


Figure 15a. Calibration curve for DMTS in DMTS-Cbi Combination

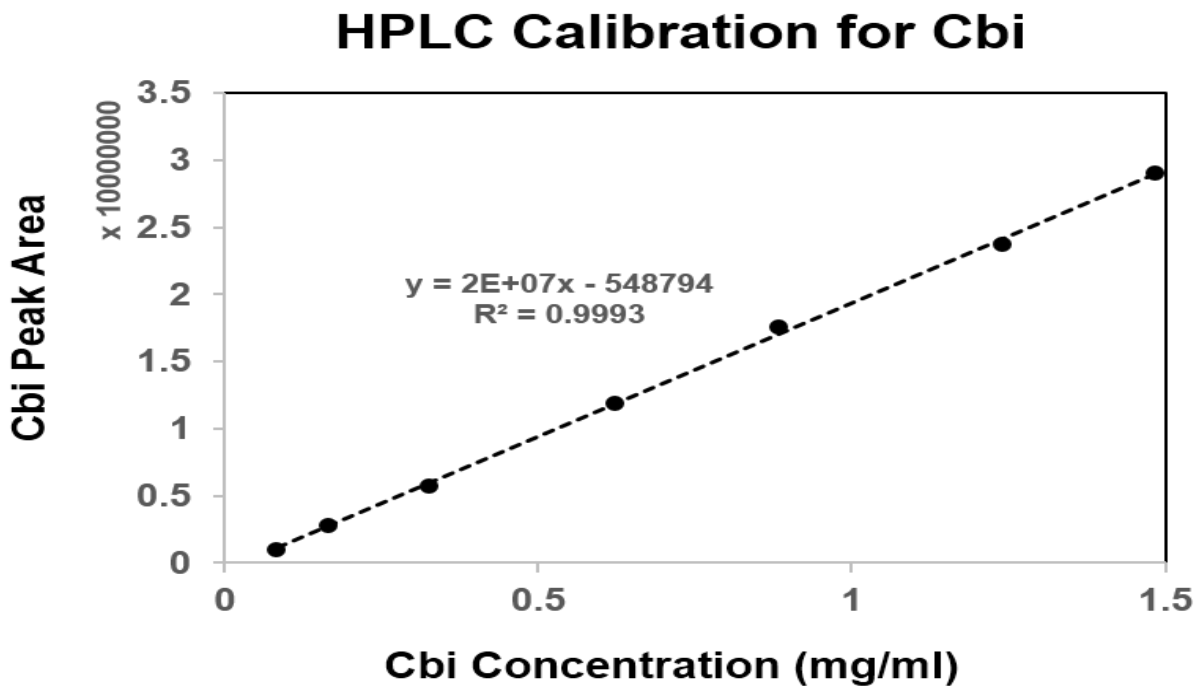


Figure 15b. Calibration curve for Cbi in DMTS-Cbi Combination

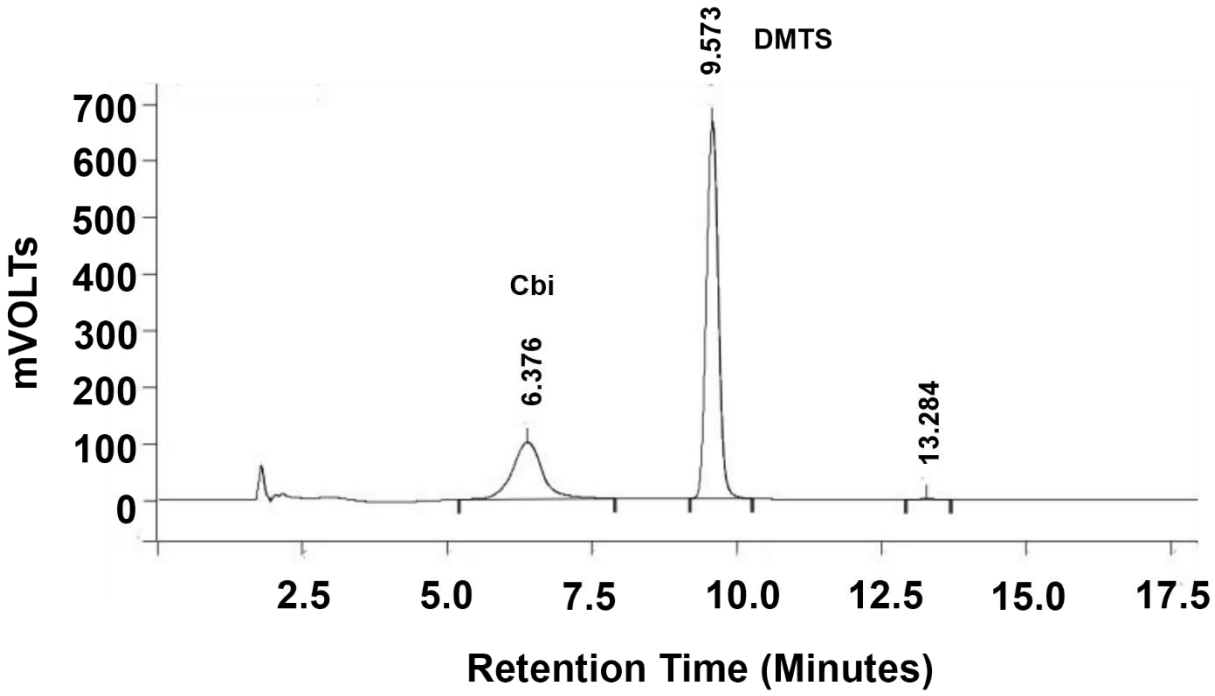


Figure 16. Representative HPLC Chromatogram for DMTS-Cbi Combination

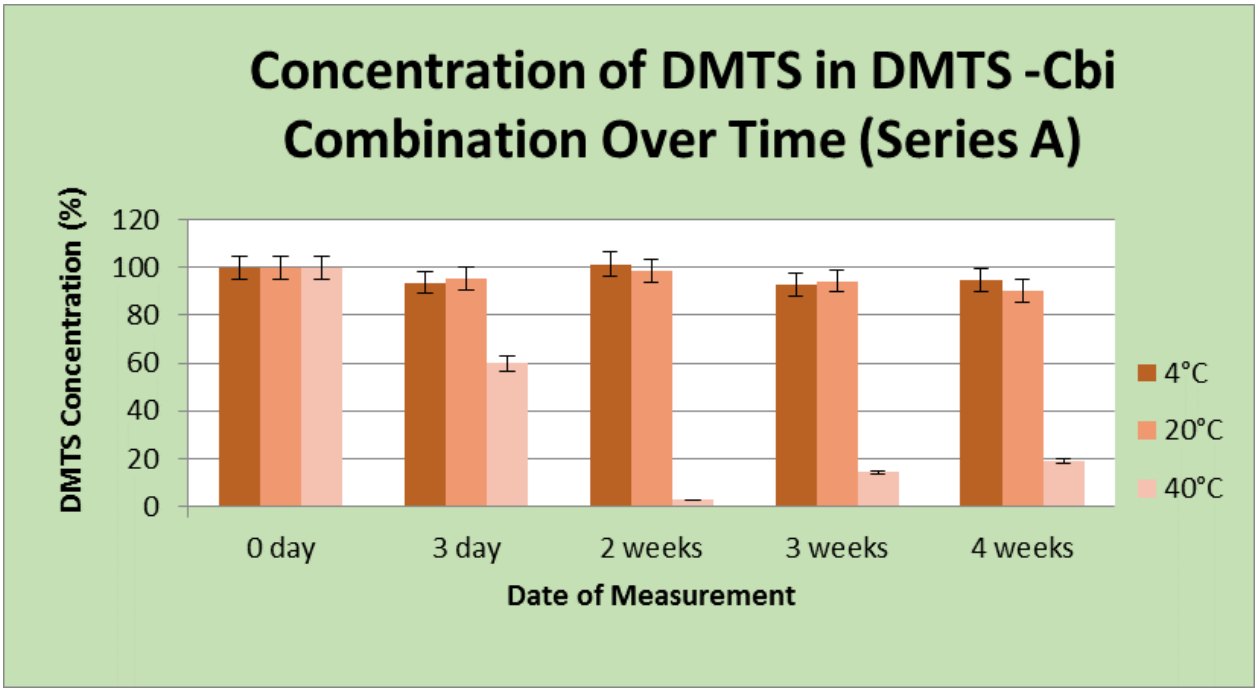


Figure 17a. In vitro Stability Results for (DMTS + Cbi) -15% Poly80 with Formulation A method

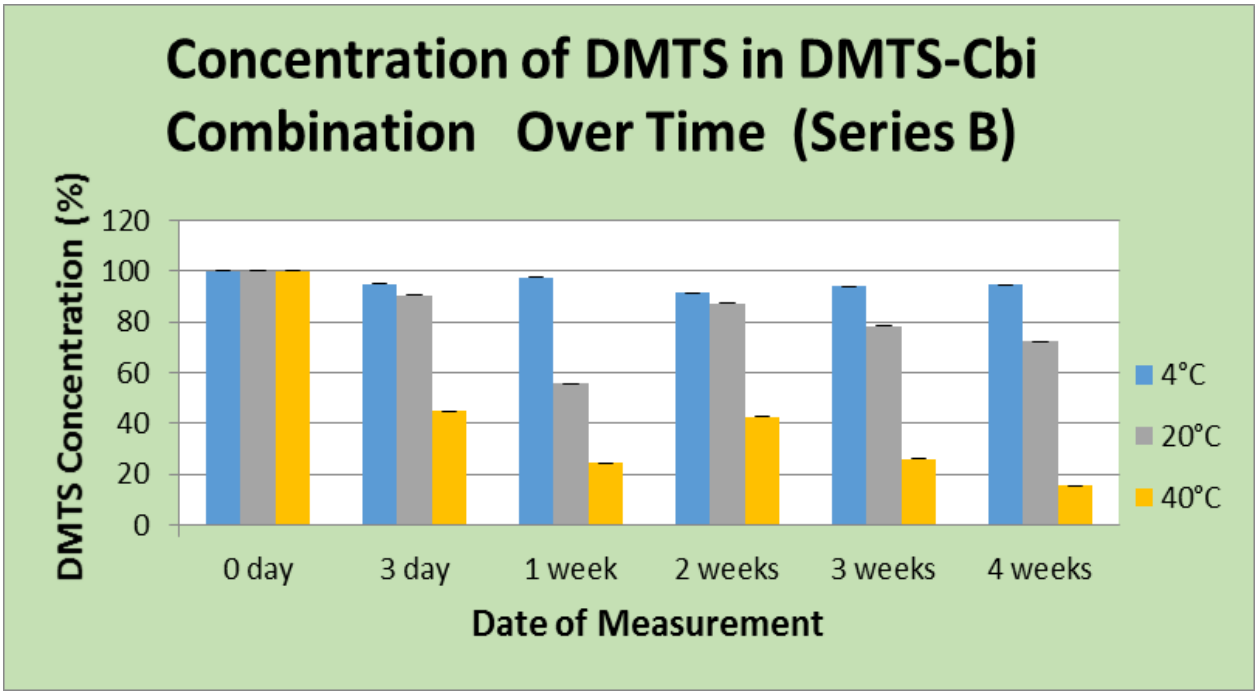


Figure 17b. *In vitro* Stability Results for (DMTS + Cbi) -15% Poly80 with Formulation B method

Picture 1. Double sealing container for sampling. (For storing larger volume samples, insert is not used).

Picture 2. Clear solutions of Poly80 –DMTS for the stability studies for the combination formulations.

Table 1: Therapeutic LD50 and APR value for mDMTS

Composition	DMTS dose (mg/kg) (im)	CN LD50 (control) mg/kg (sc)	CN LD50 in the presence of DMTS	APR
2.5 mg/mL DMTS in 26.75 mg/mL PEG_{2000}-DSPE	12.5	8.15 (6.59-10.08)	17.09 (13.97-20.92)	2.09

Table 2. Therapeutic Antidotal Protection by formulated DMTS and TS at different doses

Exp #	Treatments*	Formulation composition	APR**
1	DMTS (50 mg/kg dose) (intramuscular)	DMTS (50 mg/ml) in 15% Poly80	2.04
2	DMTS (100 mg/kg dose) (intramuscular)	DMTS (50 mg/ml) in 15% Poly80	3.4
3	DMTS (200 mg/kg dose) (intramuscular)	DMTS (50 mg/ml) in 15% Poly80	4.1
4	DMTS (100 mg/kg dose) (intramuscular)	DMTS (50 mg/ml) in 20% Poly80	3.2
5	TS (100 mg/kg dose) (intramuscular)	TS (50 mg/ml in PBS)	1.1
6	TS (200 mg/kg) (intramuscular)	TS (50 mg/ml in PBS)	1.3

***Mice received KCN (subcutaneously) one min prior to the DMTS/TS. ** APR was calculated as a ratio of LD50 of CN with and without the sulfur donors**

Table 3. Therapeutic Antidotal Protection by 20 % Poly80 –DMTS Formulated in two different ways

Exp #	CN LD50	DMTS dose (im)	DMTS Formulation	APR
1	11.22 (8.65-14.57)	0		N/A
2	33.67 (23.36-39.33)	100 mg/kg	20% Poly80*	3
3	36.43(25.95-43.69)	100 mg/kg	20% Poly80**	3.25

*DMTS was added on the same day when Poly80 was prepared
** DMTS was added on the next day

Table 4. Therapeutic Antidotal Protection by DMTS + Cbi 15% Poly80

Treatment Group	KCN LD_{50} (mg/kg)	95% confidence interval	APR
Control (NY) 1.0 mg/ml KCN	8.264	6.46-10.52	N/A
Control (NC) 1.0 mg/ml KCN	9.835	7.85-12.33	N/A
Control (NC – large) 1.0 mg/ml KCN	10.936	8.59-13.92	N/A
Control (NC) 3.5 mg/ml KCN	7.811	6.14-9.94	N/A
DMTS 100 mg/kg (NY)	29.415	22.97-35.15	3.4
DMTS 100 mg/kg (NC)	36.002	28.73-45.12	3.66
DMTS 100 mg/kg (NC –large)	33.894	27.70-41.47	3.09
Cobinamide 250 mg/kg (NC)	23.123	18.69-28.61	2.35
Cobin + DMTS (NC)*	57.656	46.01-72.25	5.862

Table 5. Therapeutic Antidotal Protection by DMTS alone with increasing DMTS concentration and (DMTS + Cbi) combinations

Recent in vivo (mouse) studies with DMTS polysorbate 80 alone and in combination with cobinamide (Cbi). Experiments conducted in January 2013 utilized the modified DMTS polysorbate 80 preparation procedures.

Test Antidote (IM)	Dose of antidote (mg/kg)	Antidote Formulation /concentration (mg/mL)	Therapeutic APR	Date
DMTS	**100**	50 mg/ml DMTS in 15% polysorbate 80	**3.1**	08/2012
Cbi	**250**	Cbi in water	**2.4**	08/2012
DMTS + Cbi	**100 DMTS/ 250 Cbi**	50 mg/ml DMTS in 15% poly 80 + Cbi in water (Injected as a cocktail)	**5.9**	08/2012
DMTS	**100**	50 mg/ml DMTS in 15% polysorbate 80	**3.9**	1/2013
DMTS	**200**	50 mg/ml DMTS in 15% polysorbate 80	**5.4**	1/2013

APPENDIX 2

EXPERIMENTAL DETAILS

A-2.1 Preparation of mDMTS

The preparation of micelles and the loading of DMTS to form mDMTS were performed in 5 consecutive steps. To optimize the manufacturing steps a number of variables were tested before the final technology was developed: Step 1: preparation of stock solutions of PEG_{2000}-DSPE with and without DMTS in ethanol. Step 2: evaporation of an aliquot of the stock solutions to form a lipid film (water bath temperature 45°C for 30 minutes followed by room temperature for 10 minutes; rotation speed: level 8; vacuum: 90 mbar; Ar gas pressure: <5 lbs/sec). Step 3: rehydration of lipid film with distilled water to yield concentrations of 1.78mM, 3.564mM, 8.91mM, 17.82mM and 26.73mM of PEG_{2000}-DSPE. Step 4: addition of excess DMTS where it was not dissolved in the stock solution. Step 5: sonication at 50°C for 20 minutes or vortexing.

The prepared samples were stored at 2-8°C for one week in sealed containers to reach equilibrium solubility and to avoid evaporation. DMTS content of the samples was measured using the HPLC-UV method described in section 2.4. Following the elaboration of the ideal preparation method micelles comprising PEG_{2000}-DSPE/TPGS (molar ratio 1:1) were prepared using the optimized method (step 1 without dissolved DMTS, step 2, step 3, step 4, step 5 with vortexing) and DMTS content was determined.

Micelles used for the animal studies were prepared using the optimized technology. Briefly, a stock solution of PEG_{2000}-DSPE was prepared in ethanol and a lipid film was formed by evaporating the organic solvent in a round bottom flask with the help of a

rotavap (Buchi Rotavapor R-210 with Vacuum controller v-855 and vacuum pump 700). The lipid film was then placed in a desiccator at room temperature till further use. For encapsulation of the sulfur donor and rehydration of the film, 2 mg/ml of DMTS and distilled water were added to the lipid film followed by vigorous vortexing for 5 minutes. The result was a translucent liquid.

A-2.2. HPLC method for DMTS determination in Micelles

An HPLC-UV method was developed for the quantitative determination of DMTS. The system consisted of a Varian Prostar 210 solvent delivery module and a ProStar UV-VIS 325 detector set at 210 nm. Injection was done manually, 20 µL of sample each time. Separation was performed on a Phenomenex Luna 5u C8(2) 100A, 250x4.6 mm 5 micron column. The mobile phase consisted of 60% acetonitrile and 40% water; flow rate 1 mL/min. Peak integration was performed using Star Chromatography Workstation Version 6.20.

A-2.3. Head-space solid phase micro-extraction-gas chromatography-mass spectrometry (SPME-GC-MS)

A manual SPME holder and fibers coated with polydimethylsiloxane (PDMS, 100 □m film thickness) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Equivalent volumes of mDMTS and DMTS in alcohol as a solvent were incubated in GC vials at 37ºC for 0, 0.5, 2, 4 and 8 h. At the end of specified incubation time, the SPME fiber was exposed to the head space of the vial for 5 min to extract DMTS. After SPME, DMTS was thermally desorbed at the GC injection port and analyzed by a FOCUS GC coupled to a DSQ II mass spectrometer (Thermo Scientific, West Palm Beach, FL,

USA). A DB-5 ((5%-phenyl)-methylpolysiloxane) capillary column (30m×0.25mm i.d., 0.25μm film thickness) was used throughout the entire experiment. Helium (99.999%) was employed as carrier gas at a constant flow rate of 1.2 mL/min. Chromatographic separations were carried out at the initial temperature held at 40 ºC for two minutes, then the temperature was ramped at 30 ºC per minute to a final temperature of 200 ºC, held for 2 minutes. The injection was achieved by inserting SPME fiber in the injection port for 2 minutes under the splitless injection mode. Temperatures of the injection port and the interface of MS detector were set at 250 ºC and 280 ºC, respectively. Electron impact (EI) was used as the ionization source.

A-2.4. Histopathology of mouse tissue after intramuscular injection of mDMTS

Mice were injected with 50, 100 and 150 μl of mDMTS intramuscularly in the caudal femoral region. Animals were sacrificed at 4, 8, 12, and 24 h post treatment and the legs were collected in 10% formalin. For histopathological studies, the tissues were trimmed, embedded in paraffin, sectioned at 4 μm and adhered to slides, routinely processed, and stained with hematoxylin and eosin.

A-2.5. Solubility studies

DMTS solubility studies were performed with co-solvents, co-solvent combinations, surfactants, surfactant combinations and cyclodextrins. Aqueous solutions of co-solvents, namely ethanol, polyethylene glycols PEG-200 and PEG-300, propylene glycol were prepared at 10, 25, 50, 75, 90%. Co-solvent combinations of the same excipients mixed at a weight ratio of 1:1 were tested at 25%, 50% and 75%. Aqueous solutions of surfactants, namely Cremophor EL, Cremophor RH 40, sodium cholate, sodium deoxycholate, polysorbate 80 were tested at 1, 5, 10, 15, 20%. Surfactant

combinations comprising Cremophor EL, Cremophor RH40 and polysorbate 80 at a weight ratio of 1:1 were tested at the same concentrations. HPβCD, RMβCD and HPγCD were all tested at concentrations ranging from 0.01 M to 0.12M.

Saturated DMTS solutions were prepared as follows: the above solvent systems were prepared in glass vials, excess DMTS was added and the samples were then vortexed (Heidolph Multi Reax, Heidolph Instruments, Cinnaminson, NJ, USA) for 20 minutes. The vials were sealed to eliminate the possibility of evaporation and kept at room temperature for equilibration. Saturated solubility was reached after 1 week. An aliquot of the samples was withdrawn and the excess DMTS was removed by centrifuging (Galaxy 20R, VWR International, Suwanee, GA, USA) at 5000 rpm for 5 minutes. The DMST content of the sample was then measured using a GC-MS or an HPLC method detailed below. In case of GC-MS measurements internal standard (1 mg/mL of dibuthyl disulfide; DBDS) was added to the samples and dilution with ethanol and cylcohexanone was performed. In case of HPLC measurements no internal standard was used and dilution of the samples was performed with acetonitrile:water in a ratio of 50:50. All samples were measured in triplicate (Figure 3).

A-2.6. GC-MS measurement for DMTS determination

A GC-MS method was applied for the quantitative determination of DMTS in co-solvent and surfactant samples. The system consisted of an Agilent Technologies 7890A GC with a 7683 autosampler and a 5975C VL MSD, triple-Axis detector (Agilent Technologies, Santa Clara, CA, USA). A DB-5MS column (30 m x 0.25 mm ID, 0.25 µm film thickness; Agilent Technologies, Santa Clara, CA, USA) was used with He carrier

gas at a flow rate of 1 mL/min and pressure of 7.6522 psi. All other parameters were identical to the ones described in Kovacs et. al. (Kovacs et al., 2013). (Figure 14a shows the calibration curve).

Extraction protocol for GC-MS measurements:

- Prepare diluted solution 1 (DS1)
  a. Take 25µl of the formulated DMTS sample and transfer it to a microcentrifuge tube.
  b. Add 375µl of 100% ethanol
  c. Add 100µl of the internal standard (1mg/mL DBDS in 100% ethanol)
  d. Vortex for 6 minutes by automated vortexer
- Prepare diluted solution 2 (DS2)
  a. Transfer 50µl of DS1 to another microcentrifuge tube (like in Step 1)
  b. Add 250µl of pure cyclohexanone
  c. Vortex the solution for 6 minutes by automatic vortexer
  d. Centrifuge for 5 minute at 5000 rpm at 4℃
- Transfer 100µl of the top layer of DS2 into a GC-MS vial containing an insert for small volumes.
- Measure on GC-MS

A-2.7. HPLC measurement

An HPLC method was used for the quantitative determination of DMTS in cyclodextrin samples. The system consisted of a Varian Prostar 210 solvent delivery module and a ProStar UV-VIS 325 detector. 20 µL of the sample was injected manually onto a Phenomenex Luna 5u C8(2) 100A, 250x4.6 mm 5 micron column. The mobile phase consisted of 85% acetonitrile and 15% water with a flow rate of 1 mL/min. Detector wavelength was 210 nm. Peak integration was performed using Star Chromatography Workstation Version 6.20. (Calibration curve is shown in Figure 14b and 15a).

Parameters and sample preparation for HPLC

- ProStar HPLC system of
  2 Solvent Delivery Modules (master and servant), Model 210,
  AutoSampler, Model 410
  UV/VIS Detector, Model 340
- Stationary phase: Phenomenex Luna 5µ C8(2) 100Å 250×4.60 mm 5 micron

- Mobil phase: Water/ACN:40/60, flow rate: 1 mL/min
- Injection 25 µL (loop 20 µL)
- UV/Vis Detector Setting: 215 nm
- Retention Time for DMTS: approximately 9.5 minutes
- Take 10 µL of formulation and add it to an eppendorph tube containing 990 µL of ethanol. Vortex for 5 minutes then inject onto HPLC for measurement.

A-2.8. Preparation of 15% Poly80-DMTS (50 mg/ml).

Detailed protocol is in Appendix 3.

A-2.9. Preparation of 20% Poly80 - DMTS (50 mg/ml)

The samples were prepared as described above for the 15% Poly80. For 20% Poly80 20 g Poly80 was dissolved in 100 ml.

A-2.10. Preparation of (DMTS+Cbi 15% Poly80) Formulation

We prepared two different formulations: Formulation A): DMTS -15%Poly80 was prepared first accordingly to the standard DMTS protocol (step1: preparing 15% Poly80 solution and equilibrating it, step 2: adding the DMTS and equilibrating again), and then we measured the required amount of Cbi and added the powder gradually to the DMTS-Poly80. After adding the Cbi, the solution was again well equilibrated before allocating the samples to a double sealed container (Picture 1). Formulation B): Both the DMTS and the Cbi were dissolved in 15% Poly80 solution in the required concentrations, and they were mixed together, and again well equilibrated by vigorous vortexing. Both formulation method resulted in clear (no cloud) DMTS solution (Picture 2 shows the two DMTS solution before Cbi was added). After Cbi was added, it was a dark red solution.

A-2.11. Stability studies with 15% Poly80-DMTS / 20% Ploly-80 - DMTS (50 mg/ml) as a function of time in double sealed container

After preparing the 25 ml of 15% Poly80-DMTS (50 mg/ml) or 20% Ploly-80 DMTS (50 mg/ml) samples in triplicates, each volumetric flask was then divided between (6x3=18) Snap-Top Vials. The vials were labeled for testing as follows: t=0 day, t=3 days, t=6 days, t=9 days, t=22 days, t=31 days with three temperature storages of + 4 °C, +20 °C and +40 °C. The vials were filled completely, each vial holding slightly over 2 mL. The lids of the vials were then snapped on and each vial was placed inside an 8 mL glass vial. The rubber lid was then placed on the vial and the vials were crimped closed, not to be opened until their day of testing. On the testing day, the vials were opened and tested by HPLC. Each vial was opened and measured only once on its specific test day. (Picture 1).

A-2.12. Stability Test for (DMTS + Cbi 15% Poly80) Combinations

Formulation A: Combination of powder Cbi with formulated DMTS solution

A solution of 15%wt poly80 in DI water was made and enough of the solution was added to make a 50mg/ml solution of DMTS in poly80. The solution was shaken for one hour and set aside. Meanwhile, 625mg of Cbi was weighed out. 5ml of the DMTS solution was added to the Cbi for a final concentration 50mg/ml DMTS and 125mg/ml Cbi in 15%poly80 solution. 100µl of the solution was added to spring inserts inside of 1.5ml auto sampler vials with screw cap septa. Each of the vials were placed into 5ml crimp cap vials and stored according to the protocol. (Temperatures: 4°C, 20°C, 40°C; sampling times: Day0, Day3, Week1, week 2, week 3 and week4). Each sampling was taken from a separate double sealed vial. Each of the samples was analyzed on the

appropriate day using HPLC. The samples were prepared by adding 10µl of the sample to 990µl of ethanol. Each sample was done in triplicate.

Formulation *B: Combination of formulated DMTS with Formulated Cbi*

A solution of 65mg/ml DMTS in 15% poly80 was made the same as in *Series B*. A solution of Cbi was prepared by dissolving powder Cbi into 15% poly80. The two solutions were mixed together to give final concentrations of 50mg/ml DMTS and 85mg/ml Cbi in 15% poly80. (There was some solubility issues with Cbi to make a higher concentration of Cbi -15% Poly80 solution). The final solution was stored the same as *Series A* and analyzed at 0 days, 3 days, 1.5 weeks, 2.5 weeks, 3.5 weeks, and 4.5 weeks. The samples were analyzed according to the same specifications.

A-2.13. Pharmacokinetics with 15% Poly80-DMTS

The primary instruments used for the experiments were a Varian ProStar HPLC (Model 210) with a UV detector (Model 340). A Centrifuge (Galaxy 20R, VWR International), Sonicator (Symphony^{TM}, VWR International), a fixed speed mini vortexer (VWR International), a Heidolph Multi Reax mechanical shaker (Heidolph Instruments), Micropipettes (Thermo Scientific), Surgical dissection kit (Carolina Biological Supply Co.) and a rat holder (Kent Scientific Corporation) were also used for these experiments. Precellys Lysing Kits having ceramic beads with average diameter of 1.4 mm were employed in conjunction with a Precellys 24 tissue homogenizer (Bertin technologies, Rockville, MD).

The following solutions were prepared freshly before the pharmacokinetic experiments

A 50 mg/mL lock solution was prepared by dissolving 1.25 g of sodium heparin in 25 mL of 5% w/v aqueous dextrose solution. A 0.9 w/v aqueous saline solution was prepared

by dissolving 22.5 g of NaCl in 25 mL of distilled water. An 80 mg/mL heparin solution was prepared by dissolving 2.00 g of sodium heparin in 25 mL of distilled water. A 15% w/w aqueous polysorbate 80A (Poly80) solution was prepared. A DMTS stock solution was prepared by dissolving DMTS into the Poly80 solution at a concentration of 47 mg/mL.

Animals used for the pharmacokinetic experiments

Male rats (250 – 300 g, Charles River Breeding Laboratories) with catheters implanted on the jugular vein were housed at room temperature in a light controlled room (22 ± 2 ˚C, 12 hours light/dark cycle). The rats were furnished with water and Teklad Rodent Diet (W) 8604 (Teklad HSD, WI) *ad libitum*. Rats were handled in accordance with The Guide for the Care and Use of Laboratory Animals and accredited by American Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. These experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Sam Houston State University.

Blood collection from catheter implanted rats

Syringes, needles and collecting tubes were sterilized and rinsed with a small volume of heparin. The rat was placed in the rat holder. The plug of the catheter was carefully removed using a pair of tweezers. The lock solution was drawn out using a heparinized syringe until blood appeared in the catheter. The required amount of blood was collected to a new heparinized micro centrifuge tube. After collecting the blood sample, the same volume of saline was administered to the rat followed by a 100 µL volume of lock solution injection. The blood samples were kept in micro centrifuge tubes and stored at 4˚C until analysis.

Addition of DMTS to rat blood

Aliquots of the DMTS stock solution were subsequently diluted with appropriate amounts of Poly80 solution to yield standard solutions having DMTS concentrations of 0.12, 0.36, 0.60, 0.84, 1.08, 2.40, 3.60, 6.00, 8.40, 12.00, 14.40 mg/ml. A 100 µL aliquot of the first standard solutions was then added to 1100 µL of intact rat blood (approximately 12 hours old and refrigerated at 4°C) in a heparinized micro centrifuge tube. This process was repeated with each successive standard to obtain eight blood samples spiked, respectively, with DMTS at concentrations of 0.01, 0.03, 0.05, 0.07, 0.09, 0.20, 0.30, 0.50, 0.70, 1.00 and 1.20 mg/mL. Each sample was centrifuged (4°C, 13 500 rpm) for 10 minutes. The plasma portion was carefully removed using a micro pipette and discarded. The sedimented portion containing blood cells was sonicated at +4 ^{0}C for 10 minutes and vortexed for 30 seconds.

Extraction of DMTS from rat blood

The sedimented portion of the blood sample was extracted with 400 µL of cyclohexanone. To facilitate the partitioning of DMTS the extraction mixture was vortexed for 30 seconds, shaken for 5 minutes (2045 rpm, 3mm orbit) and vortexed for 5 minutes. To ensure good separation of the organic and aqueous layer the extraction mixture was centrifuged (4°C, 13 500 rpm) for 10 minutes. The samples were kept at 4˚C for 2.5 hours. Approximately 40 µL of the upper cyclohexanone layer was transferred into a screw cap vial containing a Polyspring insert. A 25 mL aliquot drawn from this vial was analyzed via HPLC. A non-polar silica based HPLC column was used in this study with a Phenomenex Luna stationary phase consisting of bonded octane units with an average pore size of 100Å and an average particle size of 5µm. The

column dimensions were 250×4.60 mm. The mobile phase was a mixture of water and acetonitrile in the ratio of 40:60. The flow rate of the mobile phase was 1 mL/min and the column pressure was 100 -105 bars. Injection volume of samples was 25 µL. DMTS was detected using ultraviolet absorbance at a wavelength of 215nm.

A-2.14. Therapeutic *in vivo* experiments with mDMTS formulations

For therapeutic experiments, mDMTS (12.5 mg/kg) was administered intramuscularly after CN exposure. CN was injected subcutaneously in all experiments. LD50 values were determined by the up and down method (simulated up and down study)(Dixon, 1965). In detail, mDMTS was injected intramuscularly into the rear right leg of the mouse 30 seconds after the injection of an initial dose of KCN. The mice were then inspected if they stayed alive or died, and the same procedure was repeated with a higher or a lower dose of KCN. This pattern was followed until the stopping conditions were met (determined by the computer software program, “Implementation of Dixon & Massey UDP, Introduction to Statistical Analysis”, 1983, pp. 434-438), meaning that enough data were collected to determine the LD50 values. 10 animals were used for the LD50 determination. Injection volumes of KCN solution and mDMTS ranged from 84 to 162 µl and 144 to 197 µl, respectively. The following formula was used for the calculation of the antidote potency ratio (APR): APR = LD50 of CN with the antidote(s)/LD50 of CN without antidote(s) (control). (Results are shown in Table 1.)

A-2.15. Therapeutic *In Vivo* Efficacy Studies with (15% Poly80-DMTS)

To determine the LD50 value and therapeutic antidotal potency of the drug *in vivo* animal studies were conducted using CD-1 male mice (18–28 g; Charles River Breeding Laboratories, Inc., Wilmington, MA). Animal procedures were conducted in accordance

with the guidelines by The Guide for the Care and Use of Laboratory Animals (National Academic Press, 2010), accredited by AAALAC (American Association for the Assessment and Accreditation of Laboratory Animal Care, International). The mice were fed with water and 4% Rodent Chow (Teklad HSD, Inc., Madison, WI) ad libitum and were housed at 21°C and in light-controlled rooms (12-h light/dark, full-spectrum lighting cycle with no twilight). At the termination of the experiments, surviving animals were euthanized in accordance with the 1986 report of the AVMA Panel of Euthansia. In order to determine the LD50 value of KCN in the presence of DMTS the up-and-down method (Dixon, 1965) was used and the estimated 95% confidence interval was calculated. First the LD50 of KCN was determined using a KCN stock solution of 3.5 mg/kg in distilled water, then the LD50 was determined in the presence of the antidote (50 mg/ml DMTS in polysorbate 80 solution). The control experiment was conducted as follows: the mice were weighed and injected with an initial dose of CN subcutaneously. The mouse was observed and based on whether it lived or died a higher or a lower dose was administered to the next mouse. This was repeated until enough data was gathered to calculate the LD50. The DMTS test was conducted as follows: a mouse was weighed and injected with an initial dose of KCN solution subcutaneously, then within one min they were injected with a given dose of DMTS intramuscularly into the right rear leg. Based on the observation that the mouse lived or died a higher or lower dose of KCN was administered to the next mouse while the dose of DMTS was kept constant throughout the experiment. This was repeated until enough data was gathered to calculate the LD50. Therapeutic antidote ratio (APR) was calculated using the following formula:

$$APR = \frac{cyanide\ LD50\ with\ antagonist}{cyanide\ LD50\ without\ antagonist}$$

For each experiment 7-11 animals were used, the KCN injection volumes for the control tests were 53-77 µL and 51-134 µL for the test DMTS test. DMTS injection volumes were 36-42 µL and 71-85 µL for the 100 mg/kg and 200 mg/kg dose tests, respectively. The injection volumes of the control were held similar to the injection volumes for the test groups. KCN stock solutions: for control: 1 mg/ml (prepared freshly from the stock of 3.5 mg/ml by dilution) for the test groups: 3.5 mg/ml (prepared freshly). CN doses for control was 8-13 mg/kg, CN doses for the test groups were 25-40 mg/kg.

A-2.16. Therapeutic *In Vivo* Efficacy Studies with (20% Poly80-DMTS)

The formulation was prepared as described earlier. There two DMTS formulations tested when studies the 20% Poly80 formulation: 20% Poly 80 “A”: DMTS was added on the same day when Poly80 was prepared; 20% Poly 80 ”B”: DMTS was added on the next day. The *in vivo* efficacy tests were run the same way as described above according to the standard protocol.

A-2.17. Therapeutic *In Vivo* Efficacy Studies for (DMTS +Cbi 15% Poly80-DMTS)

The *in vivo* efficacy tests were run the same way as described above according to the standard protocol. The DMTS -15%Poly80 formulation was prepared accordingly to the Formulation method A, where first the 15% Poly80 –DMTS (50 mg/ml) was prepared, than the Cbi was added as a powder to make the final Cbi concentration of 125 mg/ml. The dose for DMTS was held as 100 mg/kg, and for Cbi = 250 mg/ml. The cocktail was injected into one leg of each mice (IM) one minute after (SC) injection of CN.

APPENDIX 3

DETAILED EXPERIMENTAL PROTOCOLS

A-3.1. Detailed Experimental Protocol for 15% Poly80- DMTS (50 mg/ml) Formulation Preparation and Analysis by GC-MS

A-3.1.1. Formulating a DTMS sample

I will give the exact report of the solution I prepared earlier today. I was preparing 35 mL of solution.

1. **<u>pH measurements</u>** of water, 15% polysorbate 80 and DMTS-Poly80.
   The pH of the DI water that will be used in the formulation was measured to be 7.434.
   The pH of the 15%Polysorbate 80 was also measured and found to be 10.019.
2. **<u>Prep a 15%(w/w) Polysorbate 80 solution</u>**
   Since I needed at least 35 mL of 15% Polysorbate 80 solution, I made 40 mL to make sure that I had enough.
   a. First, I weighed out 6 g of Polysorbate 80. (fig.1)
   b. I then added 34 g of DI water. (fig. 2,3)
   c. I swirled and shook the mixture until all of the Polysorbate 80 was dissolved and the solution was clear. (fig. 4). (Best if you let it stay at room temperature for 1-2 days)
   d. I measured the pH of the 15% Polysorbate 80 solution and found it to be 7.309.

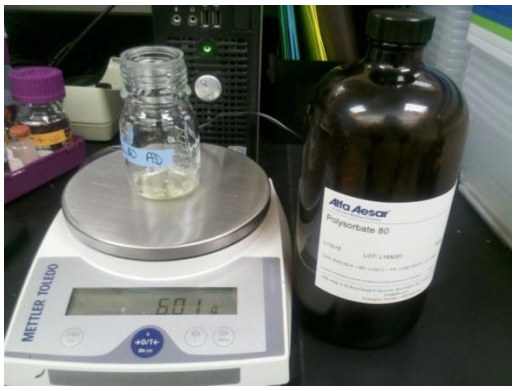


fig. 1. Weighing of Polysorbate 80

fig. 2. Weighing of water

fig. 3. Polysorbate 80 in water prior to swirling and shaking

fig. 4. Clarified Polysorbate 80 solution following swirling and shaking

3. **<u>Preparation of a 50mg/mL DMTS solution</u>**.
   a. Since I want a final DMTS concentration of 50 mg/mL, I weighed out approximately 1.25 g of DMTS into a 25 mL volumetric flask as can be seen below with my exact measurement.

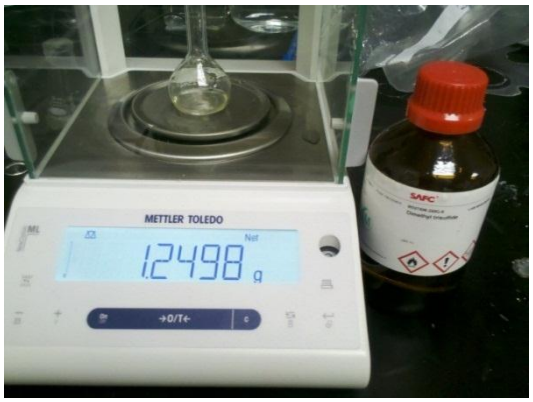


fig. 5.

   b. To the DMTS in the 25mL volumetric flask was 15% Polysorbate 80 solution was added to fill it up to the mark. To reduce the bubbles, the 15% Polysorbate 80 solution was carefully added to the DMTS.

c. I then hand-vortexed the solution (fig. 6). I used the hand-vortex because I find that it works much better and faster than our automatic vortex. At first, the solution will appear very cloudy (fig. 7), then it will gradually get a little clearer. The solution must be vortexed (vigorously shaking) at least 30-60 mins. Every once in a while, stop vortexing and let the solution settle for a bit. Settle on the table for a bit until the small bubbles disappear. I find that the best way to tell if all of the DMTS is dissolved is to look through the top into the bottom of the flask. If there is a mist of "bubbles" on the bottom, continue vortexing. If there is no mist and it appears completely clear, the DMTS is dissolved. I usually vortex for another 5 minutes just to make sure it is dissolved (I tried to take pictures of the mist but the pictures did not turn out well). A well-vortexed solution should look like the one shown in fig. 8. When it transferred to the vial (Figure9), sometimes it appears still foggy. This case you keep going with vortexing. It also can help if you put your slightly cloudy solution (fig. 9) into a refrigerator for sitting there overnight, and the next day you let it worm up until room temperature, and vortex it again for a few mins. It should clear up by then.

d. I measured the pH of the 50 mg/mL DMTS in 15% Polysorbate 80 solution and found it to be 7.688.

THE MOST IMPORTANT PARAMETERS TO BE CARE ABOUT:

- ***Vortex the polysorbate 80 solution well, it is even better if you keep it overnight to equilibrate on room temperate (Do not put it into Refrigerator before you add the DMTS)***
- ***When you prepare the DMTS solution, be sure if it is vigorously vortexed (shaken), and if it is not clear, keep going with the vortexing. You can also put the cloudy DMTS formulation into refrigerator for overnight, and after it worms up to room temperature, you keep going with the vortexing until it clears up.***
- ***When you store the samples more than one day, it should be double sealed: the best if you flush out the Oxygen from the outer container with Nitrogen, or Helium, or Argon. (Never flush out the DMTS samples: it can evaporate)***
- ***If you don't mix well the solution during the formulation preparation, (if you stop when it is still cloudy) it will precipitate later***

e. Once the solution is dissolved (cleared up), it can be put into the vials. I took pictures of each kind of vial and lid we use. First, I put as much DMTS solution as I can into the Screw Cap vial (fig. 10). I fill it as full as possible to reduce the amount of air in the vial. Next, I put a cap on the Screw Cap Vial (fig. and fig. 12)

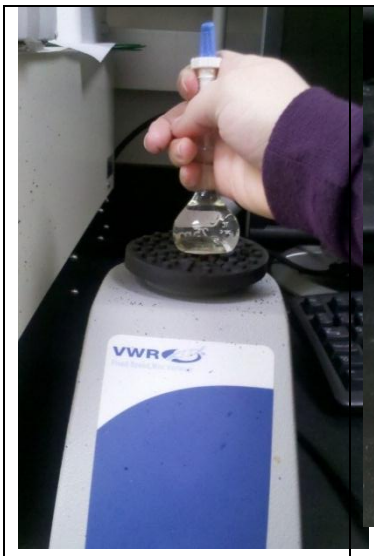


fig. 6.

fig. 7.

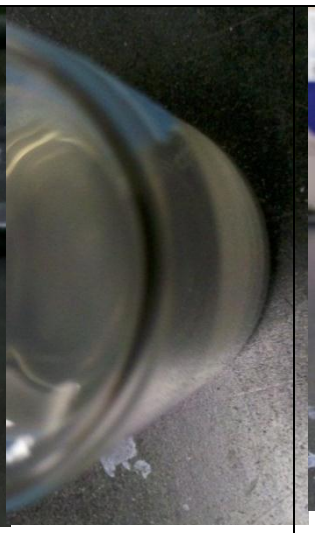


fig. 8.

fig. 9.

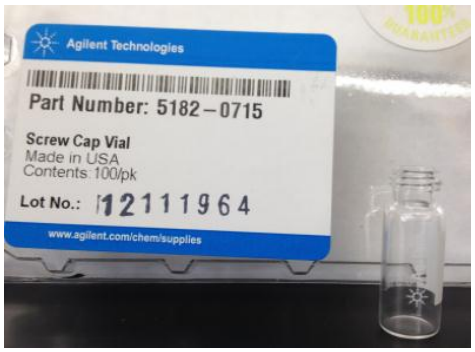


fig. 10. Screw Cap Vial (1.5 mL.)

fig. 11. Caps for Screw Cap Vial

fig. 12. Capped Screw Cap Vial

f. The Screw cap vial is then placed in a crimp vial (

g. fig. 13). The crimp vial is then stoppered with a rubber snap-on stopper (fig. 14).

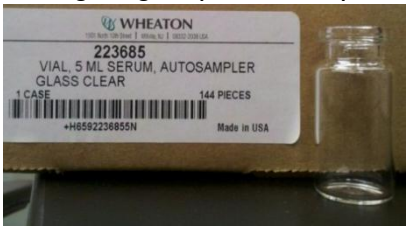


fig. 13. Crimp vial (5 ml)

fig. 14. Red rubber stopper for crimp vial

fig.15. Capped Screw Cap Vial Within a Crimp Vial

h. Finally the crimp vial is crimped closed. (The crimp caps that we use are shown in fig. 15). Fig. 17. shows the Capped Screw Cap Vial inside a Crimped and stoppered Crimp Vial.

fig. 15. Crimp Caps for serum vials

fig. 16. Capped Screw Cap Vial inside a crimped and stoppered Crimp Vial.

For a 50-200 ul samples, use an insert within the capped Screw Cap Vial (see below)

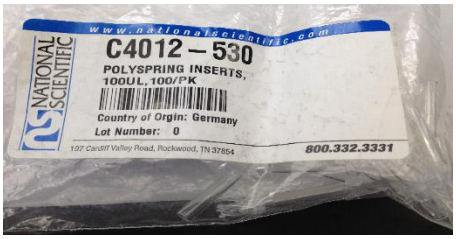


Fig. 18. For smaller volume samples **stability studies with 100 ul samples)**

fig. 19. Insert with samples **within a crimped and stoppered Crimp Vial**

fig. 20. Capped Screw Cap Vial **(e.g. inside a crimped and stoppered Crimp Vial with insert in it**

A-3.1.2. Preparing DMTS sample for GC-MS Analysis

- **Prepare diluted solution 1 (DS1)**
  e. Take 25µl of the formulated DMTS sample and transfer it to a microcentrifuge tube. (**Error! Reference source not found.**)
  f. Add 375µl of 100% ethanol
  g. Add 100µl of the internal standard (1mg/mL DBDS in 100% ethanol)
  h. Vortex for 6 mins by automated vortexer

- **Prepare diluted solution 2 (DS2)**
  a. Transfer 50µl of DS1 to another microcentrifuge tube (like in Step 1)
  b. Add 250µl of pure cyclohexanone
  c. Vortex the solution for 6 minutes by automatic vortexer
  d. Centrifuge for 5 minute at 5000 rpm at 4℃

- **Transfer** 100µl of the top layer of DS2 into a GC-MS vial containing an insert for small volumes

  - **Measure on GC-MS**

A-3.1.3. Calibration Preparation Method 1 (Original)

I. Prepare 10 calibration solutions that have DMTS concentrations spanning the concentration range of the formulated DMTS (2.5to 80 mg/mL)

80mg/mL DMTS Caliber

a. Weigh out 0.8 g of DMTS into a 10 mL volumetric flask.
b. Add ethanol to the volumetric flask to the mark
c. Vortex the solution in the volumetric flask for about 5 minutes until the DMTS is completely dissolved and transfer it to a 5 mL serum vial (shown above) labeled “80 mg/mL” and cap with the rubber cap shown above.

70 mg/mL DMTS Caliber

a. Open the “80 mg/mL” caliber solution and as quickly as possible, transfer 7 mL to a new labeled as “70 mg/mL” 5 mL serum vial. Cap the “80 mg/mL” solution immediately after transfer.
b. Add 1 mL of ethanol to the “70 mg/mL” vial and cap it. Vortex it for 30 seconds to mix.

60 mg/mL DMTS Caliber

a. Open the “70 mg/mL” caliber solution and as quickly as possible, transfer 6 mL to a new labeled as “60 mg/mL” 5 mL serum vial. Cap the “70 mg/mL” solution immediately after transfer.
b. Add 1 mL of ethanol to the “60 mg/mL” vial and cap it. Vortex it for 30 seconds to mix.

50 mg/mL DMTS Caliber

a. Open the “60 mg/mL” solution and as quickly as possible, transfer 5 mL to a new labeled as “50 mg/mL” 5 mL serum vial. Cap the “60 mg/mL” solution immediately after transfer.
b. Add 1 mL of ethanol to the “50 mg/mL” vial and cap it. Vortex it for 30 seconds to mix.

40 mg/mL DMTS Caliber

a. Open the “50 mg/mL” solution and as quickly as possible, transfer 4.8 mL to a new labeled as “40 mg/mL” 5 mL serum vial. Cap the “50 mg/mL” solution immediately after transfer.
b. Add 1.2 mL of ethanol to the “40 mg/mL” vial and cap it. Vortex it for 30 seconds to mix.

30mg/mL DMTS Caliber

a. Open the “40 mg/mL” solution and as quickly as possible, transfer 4.5 mL to a new labeled as “30 mg/mL” 5 mL serum vial. Cap the “40 mg/mL” solution immediately after transfer.
b. Add 1.5 mL of ethanol to the “30 mg/mL” vial and cap it. Vortex it for 30 seconds to mix.

20mg/mL DMTS Caliber

a. Open the “30 mg/mL” solution and as quickly as possible, transfer 4 mL to a new labeled as “20 mg/mL” 5 mL serum vial. Cap the “30 mg/mL” solution immediately after transfer.
b. Add 2 mL of ethanol to the “20 mg/mL” vial and cap it. Vortex it for 30 seconds to mix.

10mg/mL DMTS Caliber

a. Open the “20 mg/mL” solution and as quickly as possible, transfer 2.25 mL to a new labeled as “10 mg/mL” 5 mL serum vial. Cap the “20 mg/mL” solution immediately after transfer.
b. Add 2.25 mL of ethanol to the “10 mg/mL” vial and cap it. Vortex it for 30 seconds to mix.

5 mg/mL DMTS Caliber

a. Open the “10 mg/mL” solution and as quickly as possible, transfer 2 mL to a new labeled as “5 mg/mL” 5 mL serum vial. Cap the “10 mg/mL” solution immediately after transfer.
b. Add 2 mL of ethanol to the “5 mg/mL” vial and cap it. Vortex it for 30 seconds to mix.

2.5 mg/mL DMTS Caliber

a. Open the "5 mg/mL" solution and as quickly as possible, transfer 1.75 mL to a new labeled as "2.5 mg/mL" 5 mL serum vial. Cap the "5 mg/mL" solution immediately after transfer.
b. Add 1.75 mL of ethanol to the "2.5 mg/mL" vial and cap it. Vortex it for 30 seconds to mix.

II. Dilute the DMTS caliber solutions for GC-MS analysis

Prepare each of the ten DMTS caliber solutions for GC-MS analysis by carrying out the 3 steps listed below. (Note that this is the same dilution procedure that was used to prepare the formulated DMTS sample for GC-MS analysis)

1. Prepare diluted caliber solution 1 (DC1)
   a. Take 25µl of the sample and transfer it into a 1.7mL microcentrifuge tube (VWR 87003-294).
   b. Add 375µl of 100% ethanol
   c. Add 100µl of the internal standard (1mg/mL DBDS in 100% ethanol)
   d. Vortex for 6 minutes by automated vortexer
2. Prepare diluted caliber solution 2 (DC2)
   a. Transfer 50µl of DC1 to another microcentrifuge tube (as in Step 1)
   b. Add 250µl of pure cyclohexanone
   c. Vortex the solution for 6 minutes by automatic vortexer
   d. Centrifuge for 5 minute at 5000 rpm at 4 ^{o}C
3. Transfer 100µl of the top layer of DC2 into a GC-MS vial containing an insert for small volumes.

III. Measure each prepared diluted DMTS caliber on GC-MS

GC Parameters:

Agilent Technologies **GC-MS** equipment of 7890A GC system; 5975C VL MSD with Triple-Axis Detector 7693 Autosampler

Agilent J&W GC Column: **HP-5ms**
Part Number **19091S-433** ID (mm) **0.25** Length (m) **30** Film (µm) **0.25**
Ramp: **200 °C**
Flow rate: **1 mL/min**
Injection: **1 µL**, Split ratio: **20/1**
Temperature Program:
Inlet: 180°C
50°C for 2 min then
20°C/min until 250°C hold for 5 minute

A-3.2. Detailed Experimental Protocol for Therapeutic *In Vivo* Efficacy Studies:

A-3.2.1 ICD Visit to SHSU Experiment:

Purpose of the study:

- Determining LD50 data employing the Up-and-down method, and calculating APR for the pre-formulated SDX, and Cbi (UCSD, Dr Boss), and the combination of the two.
- Comparing the controls determined on mice (same species, same company, different location), to rule out the deviations of the two animal sources (NY, NC).
- Demonstrating our injection method, and all the details about how we plan, run and present the results.
- After the experiments are finished, Dr. Booker will inject some animals to compare our injection method with his one.
- Comparing the in vivo antidotal efficacy of the formulated SDX and Cbi alone and in combination, to decide if there is any advantage of going to the direction of this combination.

Experimental setup/ Solution preparations, Formulation preparation.

- Preparing solutions: Dr Petrikovics prepares the KCN stock solutions (3.5 mg/ml and 1 mg/ml) in isotonic saline solution, pH of the KCN solution 11.0+/- 0.2.
- Graduate student Siva Angalakurthi determines the CN content spectrophotometrically to make sure it is constant for each study.
- Dr. Kovacs will prepare the formulation for the test SDX molecule in Polysorbate 80 (the day before the experiment)
- Animals are held in the temperature controlled room, and those animals that will be injected in the morning, will start fasting from 6 pm and those who will be injected pm, will start fasting from 11pm (the day before the next day experiment). This way the morning batch of animals will be fasting the same time interval as the afternoon batch of animals.
- Food will be removed by student Senan Rasheed
- Mario Jane weights the mice and labels them.
- Preparing “observation room” for each animal separately and labeling as Group 1-7; Stage 1-10 (or up to 15, depending on the need to reach the stopping condition)
- Injection will be done by Stephen Lee/Mario Jane.
- Computer software will be handled by Dr. Kovacs.
- Starting doses will be determined by Dr. Petrikovics and Dr Kovacs
- Within one min after injecting the KCN solution (sc), the test antidote solutions will be injected (im).

- After injection, each animal will be placed into the prepared observation room, and each animal will be observed for half an hour before determining the next dose (up if alive, down if dead).
- Dead animals are removed from the observation room immediately and placed into a plastic bag, and stored in a refrigerator until disposal
- Alive animals are further observed periodically, until we finish the experiment for the day, and we check them again next day. If they would die later any time within the 24 hrs observation time, it would be recorded to the software.
  (Our experience is that with SDX, if they die, they die within 30 mins) (Only when SN was studied we found dead animals later).
- After each group reached the stopping conditions, the experiments are finished.
- Surviving animals are placed back to their metal cage and food and water are provided again.
- Next day the surviving animals are observed again, and if it would be necessary, the data in the computer would be changed before determining the LD50.
- APR are calculated and reported
- After 24 hr observation time the surviving animals are terminated by cervical dislocation, and disposed in plastic bags.

Solution Concentrations:

KCN stock solution concentration #1:	1.0mg/mL in NaCl solution (for control)
KCN stock solution concentration #2:	3.5mg/mL in saline (for test)
DMTS stock solution concentration:	50mg/mL in 15% Polysorbate 80 + water
Cbi concentration:	112.5mg/mL in water

Groups:

Group 1	Control 1.0mg/mL with mice from NY
Group 2	Control 1.0mg/mL with mice from NC
Group 3	DMTS in 15% Polysorbate 80 (100mg/kg) with mice from NY
Group 4	DMTS in 15% Polysorbate 80 (100mg/kg) with mice from NC
Group 5	DMTS in 15% Polysorbate 80 (75mg/kg) with mice from NC
Group 6	Cbi in water (250mg/kg) with mice from NC
Group 7	DMTS in 15% Polysorbate 80 (100mg/kg) + Cbi in water (250mg/kg) with mice from NC
Group 8	Control 1.0mg/mL with mice from NC (larger animals)
Group 9	DMTS in 15% Polysorbate 80 (100mg/kg) with mice from NC (larger animals)
Group 10	Control 3.5mg/mL with mice from NC (larger animals)

Tbl. 1. Determination of the LD_{50} of the control group (Group 1). 1.0mg/mL with mice from NY

Weight (g)	*Dose KCN (mg/Kg)*	*Volume (µL)*	*D or L*
19.65	10	197	D
17.1	8	137	L
18.6	10	186	D
18.3	9	165	L
21.8	11	240	D
21.6	8.5	184	D
20.3	7	142	L

Tbl 2. Determination of the LD_{50} of the control group (Group 2). 1.0mg/mL with mice from NC

Weight (g)	*Dose KCN (mg/Kg)*	*Volume (µL)*	*D or L*
20	10	200	D
19.7	8	158	L
22.2	10	222	L
20.2	12	242	D
23	9.5	219	L
19.9	12	239	D
19.2	10	192	D
21.4	8	171	L

Tbl 3. Determination of the LD_{50} of the control group (Group 8). 1.0mg/mL with mice from NC (larger animals)

Weight (g)	*Dose KCN (mg/Kg)*	*Volume (µL)*	*D or L*
28.1	10	281	D
26.7	8	214	L
24.6	10	246	L
26.3	12	316	D
27.4	9.5	260	L
26.2	12	314	D
25.2	9.5	239	L

Tbl 4. Determination of the LD_{50} of the control group (Group 10). 3.5mg/mL with mice from NC (larger animals)

Weight (g)	*Dose KCN (mg/Kg)*	*Volume (µL)*	*D or L*
26	10	74	D
27.3	8	62	L
26.5	10	76	D
24.0	9	62	D
26.8	7	54	L
24.5	9	63	D
26.5	7	53	L

Tbl 5: Determination of the LD_{50} of the control group (Group 3). DMTS in 15% Polysorbate 80 (100mg/kg) with mice from NY

Weight (g)	*Dose KCN (mg/kg)*	*KCN Vol. (µL)*	*DMTS Dose*	*DMTS Vol. (µL)*	*D or L*
20.5	25	146	100	41	D
20.3	20	116	100	41	L
18.2	25	130	100	36.5	L
19.3	31	171	100	38.5	D
21	25	150	100	42	L
19.7	30	169	100	39.5	L
21.5	37.5	230	100	43	D
21.8	28	174	100	43.5	D
20.7	22	130	100	41.5	L

Tbl 6: Determination of the LD_{50} of the control group (Group 4). DMTS in 15% Polysorbate 80 (100mg/kg) with mice from NC

Weight (g)	*Dose KCN (mg/kg)*	*KCN Vol. (µL)*	*DMTS Dose*	*DMTS Vol. (µL)*	*D or L*
23.1	25	165	100	46	L
19.4	31	172	100	39	D
17.5	25	125	100	35	L
20.0	32	183	100	40	D
21.3	26	159	100	42.5	L
20.0	30	171	100	40	L
21.9	37.5	235	100	44	L
22.8	47	306	100	45.5	D

Tbl 7: Determination of the LD_{50} of the control group (Group 5). DMTS in 15% Polysorbate 80 (75mg/kg) with mice from NC

Weight (g)	*Dose KCN (mg/kg)*	*KCN Vol. (µL)*	*DMTS Dose*	*DMTS Vol. (µL)*	*D or L*
20.7	25	148	75	31	D
19.7	20	113	75	30	L
21.1	25	151	75	32	D
20.7	21	124	75	31	L
19.4	26	144	75	29	D
23.1	22	145	75	35	D
20.7	18	106	75	31	D
20.3	15	87	75	30.5	L

Tbl 8: Determination of the LD_{50} of the control group (Group 6). Cobinamide in water (250mg/kg) with mice from NC

Weight (g)	*Dose KCN (mg/kg)*	*KCN Vol. (µL)*	*Cbi Dose*	*Cbi Vol. (µL)*	*D or L*
23	20	131	250	51	D
21.2	16	97	250	47	L
22.0	20	126	250	49	L
20.8	25	149	250	46	D
23.7	20	135	250	52.5	L
21.4	24	147	250	47.5	L
21.7	30	186	250	48	D
20.2	24	139	250	45	D
21.2	19	115	250	47	L

Tbl 9: Determination of the LD_{50} of the control group (Group 7). DMTS in 15% Polysorbate 80 (100mg/mg) + Cobinamide in water (250mg/kg) with mice from NC

Weight (g)	*Dose KCN (mg/kg)*	*KCN Vol. (µL)*	*DMTS Dose*	*DMTS Vol (µL)*	*Cbi Dose*	*Cbi Vol. (µL)*	*D or L*
18.1	25	129	100	36	250	40	L
21.8	31	193	100	44	250	48.5	L
21.1	39	235	100	42	250	47	D
20.0	32	183	100	40	250	44.5	L
22.4	40	256	100	45	250	50	L
23.7	47	318	100	47.5	250	52.5	L
21.5	59	362	100	43	250	48	L
20.9	74	442	100	42	250	46.5	D
18.9	65	351	100	38	25	42	L

Tbl 10: Determination of the LD_{50} of the control group (Group 9). DMTS in 15% Polysorbate 80 (100mg/kg) with mice from NC (larger animals)

Weight (g)	*Dose KCN (mg/kg)*	*KCN Vol. (µL)*	*DMTS Dose*	*DMTS Vol. (µL)*	*D or L*
25.3	25	181	100	51	L
23.7	31	210	100	47.5	D
26.7	25	191	100	53.5	D
25.2	20	144	100	50.5	L
29.0	25	207	100	58	L
24.4	30	209	100	49	L
27.5	38	299	100	55	D
25.5	30	219	100	51	L
26.4	38	287	100	53	L
25.7	48	352	100	51.5	D

RESULTS SUMMARY:

Test	LD_{50}	APR
Control 1.0mg/mL with mice from NY	8.264	N/A
Control 1.0mg/mL with mice from NC	9.835	N/A
Control 1.0mg/mL with mice from NC (larger animals)	10.936	N/A
Control 3.5mg/mL with mice from NC (larger animals)	7.811	N/A
DMTS in 15% Polysorbate 80 (100mg/kg) with mice from NY	28.415	28.415/8.264=3.4
ADMTS in 15% Polysorbate 80 (100mg/kg) with mice from NC	36.002	36.002/9.835=3.66
DMTS in 15% Polysorbate 80 (100mg/kg) with mice from NC (larger animals)	33.894	33.894/10.936=3.09
Cbi in water (250mg/kg) with mice from NC	23.123	23.123/9.835=2.35
DMTS in 15% Polysorbate 80 (100mg/kg) + Cbi in water (250mg/kg) with mice from NC	57.656	57.656/9.835=5.862

DMTS (100 mg/kg) Average APR = **3.38**

Relative Antidotal Potency Ratio (RAPR) = **APR1/APR2**

Test	APR	RAPR
DMTS in 15% Polysorbate 80 (100mg/kg) (average)	3.38	5.82/3.38=1.72
Cbi in water (250mg/kg) with mice from NC	2.35	5.82/2.35=2.47
DMTS in 15% Polysorbate 80 (100mg/kg) + Cbi in water (250mg/kg) with mice from NC	5.82	

CFDA#: 12.420 PTN#: 13004 Funding Period

Detailed Budget for Budget Period Year 1

Start Date: 09/01/2012

End Date: 08/31/2013

Sponsor: US Army - ICSD

Project Title: Cyanide Research

Principal Investigator: Ilona Petrikovics

A. Personnel *(Applicant organization only)*		Months Devoted to Project				Dollar Amount Requested *(omit cents)*			
Name	Title	Acad. Months	Summer Months	Base Annual Salary (3% COLA)	FTE %	Salary Requested	Fringe Benefits (32%)	Matching or In-Kind Funds *(if required)*	Total
Ilona Petrikovics	PI	9	0	71,548	25.0%	$ 17,887	5,724	-	$ 23,611
Ilona Petrikovics	PI	0	3	71,548	100.0%	$ 23,849	7,632	-	$ 31,481
David Thompson			1	65,704	100.0%	$ 7,300	2,336	-	$ 9,637
							-	-	$ -
						$ -	-	-	$ -
						$ -	-	-	$ -
			SUBTOTAL →			$ 49,037	$ 15,692	$ -	$ 64,729

Student Wages *(Applicant Organization Only)*		Months Devoted to Project				Dollar Amount Requested *(omit cents)*			
Name	Role/Title on Project	Acad. Months	Summer Months	Hours Worked	Pay Rate	Wages Requested	Fringe Benefits	Matching or In-Kind Funds *(if required)*	Total
							-	-	$ -
Post-Graduate student							-	-	$ -
Undergraduate student	Project assistant					$ 3,995	306	-	$ 4,301
Post-Doc				100%	35000/yr		-	-	$ -
			SUBTOTAL →			3,995	306	-	4,301

TOTAL SALARY, WAGES, AND FRINGES →	53,032	15,997	-	69,029

B. Travel (SHSU Staff/Faculty/Students only)		Amount	Matching Funds	Total
			-	-
Air Fare		550	-	550
Ground Transportation		110	-	110
Lodging		1,956	-	1,956
Mileage		495	-	495
Parking		40	-	40
Per Diem & Incidentals		1,670	-	1,670
			-	-
		4,821	-	4,821

C. Capital Equipment *(items ≥ $5,000; IDC Exempt)*	Amount	Matching Funds	Total
		-	-
		-	-
	-	-	-
	-	-	-
Subtotals (exempted from IDC base)	-	-	-

D. Materials/Supplies/Consumables		Amount	Matching Funds	Total
Consumables lab supplies and reagents		19,661	-	19,661
Office supplies/shipping/lab equipment supplies		2,000	-	2,000
Animals/rats	$90.00 + 3.00 shipping = $93.00 x 12	1,116		
Animals/Mice	$5.80 + $2.00 shipping = $7.80 x 750 mice	5,850	-	5,850
		-	-	-
Subtotals		28,627	-	27,511

E. Other Expenses			Amount	Matching Funds	Total
		IDC Exempt	-	-	-
Contracted Services - Kristof Kovacs - Visiting scientist	100% x 1 month		7,000	-	7,000
Contracted Services - Visiting researcher	100% x 12 months		33,400	-	33,400
Conference registrations			300	-	300
Instrument Service and Maintenance Fees			3,500	-	3,500
				-	-
				-	-
				-	-
				-	-
				-	-
	Total IDC Exempted Amount	-	-	-	-
		Subtotals	44,200	-	44,200

F. Sub contractual	Amount	Matching Funds	Total
		-	-
	-	-	-
	-	-	-
Subtotals	-	-	-

Subtotal Direct Costs for Budget Period					146,677
Facilities & Administrative (IDC) Costs	IDC Applied to:	MTDC	IDC Rate:	31.5%	46,203
31.5% MTDC - On Campus, 10.2% MTDC - Off Campus	IDC Base Amount:	146,677			
Matching Funds	** Indicate % Required Here →			0%	-
Funds retained by SHSU (Amount requested from Sponsor less Sub-Contracts)					192,880
Total Requested From Sponsor					192,880
Total Project Costs					192,880

Prepared by Dee Myall 9/4/2012

TARGET: 192,880

DIFFERENCE 0

9/10/2012